The development and application of DNA metabarcoding to non-invasively assess seabird diets, using albatrosses as a model

by

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Abstract

The diets of marine organisms provide valuable insights into their behaviour, ecology, population vulnerabilities and the role they play in marine foodwebs. Many seabird populations are threatened by interactions with commercial fisheries resulting in incidental mortality or competition for resources, and global environmental changes are affecting the abundance and availability of prey. Understanding their prey requirements and dietary flexibility in this context is valuable for effective conservation and management.

Conventional studies using stomach contents analysis can be invasive and suffer considerable drawbacks such as overestimation of prey represented by hard parts and underestimation of soft-bodied prey. DNA metabarcoding of scats provides a non-invasive dietary analysis method that identifies prey DNA and overcomes some of the drawbacks of conventional methods. However, this method has rarely been used on seabirds. It is unknown whether DNA is viable in scats that have been exposed to the harsh weather typical of seabird colonies, if dietary information can be collected during all breeding stages, or whether DNA metabarcoding offers improvements over other dietary assessment methods for evaluating marine ecosystem changes and interactions with fisheries.

Albatrosses provide an ideal model to develop and apply DNA metabarcoding to seabirds. They are one of the most threatened seabird groups due primarily to human activities impacting upon breeding populations, and are used as keystone monitoring species for identifying changes in marine ecosystems. In this thesis, I describe the development and assessment of DNA dietary analysis as a non-invasive tool to evaluate and monitor threats to seabird populations posed by changing environmental conditions and interactions with commercial fisheries. To achieve this I have used albatrosses as a model to:

1) Examine the current methods used to assess diets and identify gaps in our knowledge and propose a framework for future diet monitoring studies;
2) Develop optimised scat collection protocols to ensure high quality dietary data is obtained;
3) Determine the importance of gelatinous prey in the diets of a seabird indicator species used for ecosystem monitoring; and
4) Assess the application of DNA metabarcoding to detect fishery discards in the diets of threatened seabirds across broad geographic ranges and determine implications for conservation and management.

The first component of this thesis was a systematic review of the literature to identify the methods used to assess albatross diets. I investigated the spatial and temporal application of these methods and species studied to identify knowledge gaps. Most albatross studies have focused on the chick-rearing period, and diet during other breeding phases is comparatively poorly known. There was a pronounced shift over time in the preferred method of characterising diets, from the morphological examination of prey remains to stable isotope analysis of tissue. This shift has reduced the volume of detailed taxonomic information available from morphological studies. Additionally, there are few long-term dietary datasets available. This reduction in recent prey information and paucity of long-term studies impacts our ability to monitor broader changes in marine ecosystems and has implications for management of threatened albatrosses. DNA-based dietary analysis provides a potential method to fill some of these information gaps and provide high taxonomic resolution of prey.

Shy albatross (*Thalassarche cauta*) were used as a case study to investigate how DNA amplification success and the proportion of food DNA detected are influenced by both environmental and physiological parameters. Albatross colonies are often remote and exposed; therefore it is unknown if dietary DNA can easily be obtained from scats in these conditions or during all breeding stages. A broad ranging universal PCR primer set enables identification of all major prey groups; however, this method also amplifies non-food DNA. Both the amount and type of non-target DNA varies with sample freshness, the collection substrate, fasting period and developmental stage of the consumer. I developed optimised scat collection protocols to enable high quality dietary DNA to be collected during all breeding stages. These will also minimise contamination issues from non-target DNA and provide standardised field methods in this rapidly expanding area of research.

I was able to apply DNA metabarcoding to assess the diets of black-browed albatross at seven colonies across their species range through collaboration with a global network of researchers. This circumpolar species has suffered population declines due primarily to incidental mortality from commercial fisheries. They are used as an indicator species to identify changes in the overall species composition of an ecosystem by the Commission for the Conservation of Antarctic and Marine Living Resources (CCAMLR) Ecosystem Monitoring Program. As such, they provide an ideal model species to evaluate the use of DNA metabarcoding to monitor marine based threats.

Albatross diets were examined at a low level of taxonomic resolution using universal primers to assess the importance of gelatinous prey. Diets are conventionally assessed from stomach content

analyses which cannot easily detect soft-bodied prey. Such biases may impact our detection of important ecosystem regime shifts. Fish was the main dietary item at most sites, however scyphozoan jellyfish DNA was present in 37% of samples and up to 80% of samples at some sites. Warmer oceans and overfishing of finfish are predicted to favour jellyfish populations, therefore there is a need to review dietary assessment methods used for ecosystem monitoring. Future seabird monitoring programs should be designed to detect diet changes across the full prey spectrum, including jellyfish, so any potential impact on seabird breeding success and survival can be evaluated.

Group-specific primers for bony fish were used to identify the diversity of fish prey consumed by black-browed albatross at five colonies and identify any overlaps with commercial fishery species (either target, bycatch or bait species). Across all sites, 51 fish species from 33 families were identified. There was extensive geographic variation but little inter-annual variability in fish species consumed. The prevalence of commercial fishery species detected in the diets of the albatross during the breeding season highlights that interactions and/or competition with fisheries are still ongoing for this species, particularly at the Falkland and Kerguelen Islands. This study highlights the potential value of DNA metabarcoding as a fishery resource management tool.

This body of work has shown that DNA metabarcoding of seabird scats provides a non-invasive dietary method for identifying and monitoring marine based threats, which can be applied during all stages of the breeding season. The ability to detect gelatinous prey and the high taxonomic resolution delivered means it provides a valuable alternate or complementary dietary method for ecosystem monitoring and fishery resource management. The development of DNA dietary analysis techniques described in this thesis will enable researchers and conservation efforts around the world to obtain further information on ecosystem linkages. This will enable ongoing monitoring and evaluation of marine threats to seabird populations.

Declaration of originality:

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief contains no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Julie McInnes 07/06/2017

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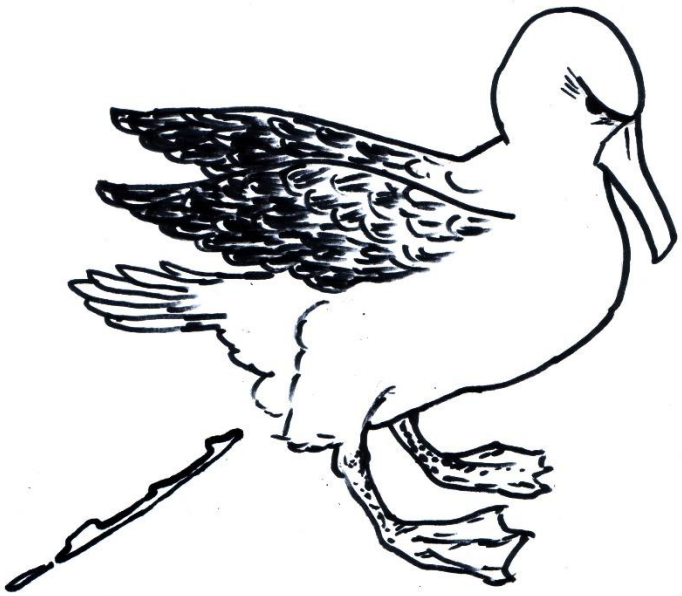

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Chapter 1 – Introduction

"The inhabitants of the watery element are made for wise men to contemplate, and for fools to pass by without consideration"

Isaak Walton

1.1 The importance of seabird dietary studies

Determining the diet of marine organisms is essential to understand their behaviour, ecology, threats and the role they play in marine foodwebs. Through direct identification of prey, or by elucidating trophic level and habitat use, dietary studies provide insights into the ecology of a species and their prey by examining foraging behaviour (Cooper et al. 1992, Hedd and Gales 2001, Ceia et al. 2012), the effects of prey availability on breeding success (Croxall et al. 1999, Arata et al. 2004), competition between sympatric species (Prince 1980, Weimerskirch et al. 1986, Cooper and Klages 1995, Waugh et al. 1999, Cherel et al. 2002), prey ecology and distribution (Clarke and Prince 1981, Imber 1992, Cherel and Weimerskirch 1999, Xavier et al. 2006), and use of discards and hence overlap with fisheries (Gould et al. 1997, Arata and Xavier 2003, Colabuono and Vooren 2007, Bugoni et al. 2010).

Understanding the diet of seabirds is particularly important to help monitor the threats that are posed by commercial fisheries and climate change (Constable et al. 2000, Chambers et al. 2011, Barbraud et al. 2012). Many commercial fisheries overlap spatially and temporally with seabird foraging areas, with some interactions leading to incidental mortality and competition for resources. Incidental mortality of seabirds can occur through interaction with both long-line (Brothers et al. 1999a, Tuck et al. 2011) and trawl fishing gear (Sullivan et al. 2006, Watkins et al. 2008). Birds are attracted to the supplementary food source provided by baits, by fish as they are hauled in, and by fish parts discarded overboard during processing. These discards can include offal of target fish species or whole non-target species. In long-line fisheries, seabirds risk being caught on hooks and drowned, whereas in trawl fisheries they risk hitting warp or sonde cables or being caught in nets. These interactions can be major drivers of population change, and incidental mortality has been linked to substantial declines in several seabird species (Weimerskirch and Jouventin 1987, Berrow et al. 2000, Nel et al. 2002a, Phillips et al. 2016). Mitigation measures are employed by many fisheries to reduce the risk of mortality. These include long-line weighting to increase bait sink rates, restricted operating times through night setting or season closures, and physical barriers during setting and hauling such as scaring lines (tori lines) and warp deflectors for trawl fisheries (Løkkeborg 2008, Pierre et al. 2014). However, the implementation rate of mitigation measures and the consequential reduction in risks to seabirds varies between jurisdictions and fisheries.

Environmental changes, primarily driven by anthropogenic carbon emissions, are causing changes to marine ecosystems through warming and increased ocean acidification (Feely et al. 2009). These changes affect marine food-webs and drive spatial and temporal changes in prey abundance and

availability (Constable et al. 2014). Marine predators depend on prey that may be patchily distributed or show seasonal variation, which may be affected by changing environmental conditions. Ocean warming may negatively impact the breeding success and survival of marine predators if they are unable to adapt to these changing conditions (Grémillet and Boulinier 2009). Reduction in prey availability or changes in distribution can cause prey switching, increase foraging duration, or alter breeding phenology to match seasonal peaks in prey abundance (Gjerdrum et al. 2003, Lea et al. 2006, Le Bohec et al. 2008, Xavier et al. 2013). Environmental change may also cause changes to fisheries, including their target species (Barbraud et al. 2012).

Dietary studies of seabirds provide a mechanism to assess environmental and fisheries-related changes in marine systems. The relative inaccessibility of the marine environment poses significant challenges to estimating biomass, abundance and distribution of marine organisms. However, seabirds are relatively accessible during the breeding season, allowing information on trophic interactions to be assessed (Block et al. 2011). Monitoring these top predators is important not only for the conservation and management of the individual species, but also provides indicators of the status of the broader marine ecosystem (Cairns 1987). The Commission for the Conservation of Antarctic and Marine Living Resources (CCAMLR) monitors eight apex predators, in the CCAMLR Ecosystem Monitoring Program (CEMP; SC-CCAMLR 1997). One important component of these programs is the collection of robust, long-term dietary datasets that can provide information on species ecology and the abundance and distribution of prey.

Seabird species breed and forage across a broad geographic range that can cover many management jurisdictions. Responsibility for conservation and management of breeding habitat, protection of food resources, and threat abatement plans require international collaboration and coordination. Two primary examples are CCAMLR and the Agreement for the Conservation of Albatross and Petrels (ACAP). ACAP encourages member nations to undertake appropriate management and monitoring of albatrosses and petrels, which enables international collaboration on knowledge of population status and trends, threats and mitigation measures and enables provision of best practice guidelines. CCAMLR takes a whole ecosystem approach to ensure sustainable fishing practices in the Southern Ocean and therefore protection of seabirds and their prey. Both ACAP and CCAMLR work towards reducing incidental mortality of seabirds in fisheries and monitoring the effects of environmental changes that might affect both seabirds and the ecosystems in which they live.

1.2 Dietary methods

Different types of dietary analysis provide alternative datasets for assessing environmental and fisheries-related changes in marine systems. Morphological analysis of stomach contents and stable isotope analysis are the main dietary methods used to assess marine predators (Duffy and Jackson 1986, Barrett et al. 2007, Bowen and Iverson 2013). Stomach content samples may be obtained from dead birds, regurgitated pellets, stomach lavage or induced regurgitation. The datasets obtained can provide high taxonomic resolution of the prey consumed, including meal sizes and prey size classes. However, stomach content analysis suffers from a predictable bias, in that entirely soft-bodied prey, such as gelatinous zooplankton and fish larvae, are much less likely to survive digestion. Hard prey remains such as cephalopod beaks, fish bones and crustacean carapaces are more likely to be represented in diet samples (Barrett et al. 2007). This issue is compounded by the retention of some prey parts in the stomach. For example, squid beaks can be retained for up to 50 days in albatrosses (Furness et al. 1984). Otoliths are calcium carbonate structures of the inner ear which differ in structure between fish species, and are typically needed to identify and estimate quantities of the fish prey consumed. However, taxonomic identification of fish can be difficult if the prey is small and digests quickly (including larvae and eggs), if the head is not eaten or if the otolith is eroded (Duffy and Jackson 1986, Barrett et al. 2007). More recent studies have used a combination of body parts to identify and quantify the fish species consumed (Cherel et al. 2000b, Colabuono and Vooren 2007); however, flesh and many body parts are not diagnostically different. Obtaining stomach content samples can also be invasive, especially using stomach lavage which involves flushing of the stomach with water (Clarke and Kerry 1994, Chiaradia et al. 2003). Induced regurgitation is less invasive as it does not require flushing and has been shown to have no long-term effect on the chick survival (Phillips 2006). However, it still deprives the chick of a meal and is considered too invasive by some management authorities (Delord et al. 2011, DSEWPAC 2011).

Stable isotope analysis measures ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ (occasionally $^{34}S/^{32}S$) in feathers, blood or other tissues from the target animal, which reflects those in their prey (Inger and Bearhop 2008). Stable isotope analyses do not suffer from the same biases associated with differential prey digestion and samples can be obtained during all breeding stages. However, stable isotope analyses lack the taxonomic resolution to identify prey to species, instead they provide information on the trophic level of prey consumed.

Dietary studies provide species occurrence data for the foraging area of the animal, allowing the overall species composition of an ecosystem to be estimated, including the availability and

distribution of different prey groups (Croxall et al. 1999, Chiaradia et al. 2010). However, the method used to collect diet data may have a limited ability to accurately identify all trophic connections. The under-estimation of gelatinous prey consumption and difficulty assessing some fish species when the otolith isn't present, or fish bones are degraded, can bias the information obtained from stomach contents (Barrett et al. 2007). Although stomach contents studies have not identified gelatinous prey to be an important dietary component, evidence is building that gelatinous prey are common in seabird diets through the use of stomach temperature loggers used to identify feeding events at sea (Catry et al. 2004), stable isotopes (Connan et al. 2014), animal-borne cameras (Sutton et al. 2015, Thiebot et al. 2016) and DNA metabarcoding of scat samples (Jarman et al. 2013, McInnes et al. 2016a).

1.3 Dietary DNA metabarcoding

DNA metabarcoding of scats is a dietary method that enables the identification of food DNA in predator scats (O'Rorke et al. 2012a, Pompanon et al. 2012). The method is non-invasive, does not suffer from biases associated with retention of hard-parts, and can detect soft-bodied prey making it ideal for use on albatross. Dietary DNA metabarcoding uses high-throughput sequencing of small, highly variable DNA regions that survive digestion to identify prey species (Pompanon et al. 2012). This may involve identification of a particular prey species using species-specific markers (Jarman and Wilson 2004), prey within a taxonomic group using group-specific markers (Jarman et al. 2004, Murray et al. 2011, Zeale et al. 2011), identification of all prey taxa using universal metazoan markers (O'Rorke et al. 2012a, Jarman et al. 2013); or a combination of these approaches (Deagle et al. 2009, Bowser et al. 2013).

Dietary DNA metabarcoding of vertebrate scat samples has been applied to a large number of land predators and herbivores (Elfström et al. 2014, Kartzinel et al. 2015, Kartzinel and Pringle 2015, Lopes et al. 2015), marine mammals (Jarman and Wilson 2004, Deagle et al. 2005, Deagle et al. 2009), but to date only a small number of seabirds including three penguin species and puffins (Deagle et al. 2007, Deagle et al. 2010, Bowser et al. 2013, Jarman et al. 2013, McInnes et al. 2016a). The popularity and application of this dietary method for seabirds is increasing rapidly. However, there is limited information on the quality and quantity of dietary information that can be obtained from seabird colonies and whether it can be collected during all breeding stages. To characterise the entire diet of an animal requires universal metazoan markers that are capable of amplifying DNA from any food species (King et al. 2008, Deagle et al. 2009, Jarman et al. 2013). However, as these markers amplify all eukaryotes, they also amplify unwanted non-food DNA, which consequently

reduces the proportion of food DNA sequences detected. Their applicability in exposed seabird colonies may also be limited because UV and rain can reduce the PCR amplification success of exposed scats (Oehm *et al.* 2011). Contamination from non-food DNA such as insects, parasites and fungi will also reduce the proportion of food DNA detected. There are currently no studies that investigate how the collection of samples impacts the detection of food DNA by universal metazoan markers.

1.4 Albatross as a model group to develop DNA metabarcoding methods

Albatrosses provide an ideal model group to develop DNA metabarcoding methods for seabirds and to test the application of these methods for monitoring changes in marine ecosystems and interactions with commercial fisheries. Albatrosses are one of the most threatened seabird groups, with population declines persisting for many species (Phillips et al. 2016). Of the 22 currently recognised species, 16 are on the International Union for Conservation of Nature (IUCN) Global Red List and the other six species are classified as near-threatened (IUCN 2015; Table 1.1). They are threatened primarily by human activities, both in the marine environment and at their breeding sites on land. Two of the greatest threats facing albatross populations are interactions with commercial fisheries and global environmental changes (Croxall et al. 2002, Chambers et al. 2011).

Albatrosses are a wide-ranging group that spend the majority of their lives at sea, typically only returning to land to breed. During the breeding season, this provides the opportunity to collect dietary samples and develop DNA metabarcoding methodologies. However, they pose potential issues for DNA dietary metabarcoding. Albatross are known to undertake both short and long distance foraging trips (Weimerskirch et al. 1994, Weimerskirch et al. 1997a), and foraging trip duration can vary between breeding stages (Stahl and Sagar 2000). It is unknown how fasting may affect the amount of food DNA recovered from scat samples and if DNA dietary analysis can be used during all breeding stages. Furthermore, albatross colonies are often remote and access can be difficult or infrequent. It is important to understand how environmental and physiological variables may affect the quality and quantity of dietary data to ensure sample sizes and sampling timing are adequate.

1.5 Thesis aims

The overall aim of this thesis research is to determine if DNA metabarcoding of scats will provide a useful, non-invasive tool to assess seabird diets and enable continued and improved evaluation and

monitoring of marine ecosystems and interactions with commercial fisheries. To achieve this, I used albatross as a model group to:

1. Examine the current methods used to assess diet and identify gaps in our knowledge.
2. Investigate how DNA amplification success and the proportion of food DNA detected are influenced by both environmental and physiological parameters.
3. Determine the importance of gelatinous prey in the diet of a seabird indicator species used for ecosystem monitoring.
4. Assess the application of DNA metabarcoding to detect fishery discards in the diet of threatened seabirds across broad geographic ranges and determine implications for conservation and management.

1.6 Study species

Two albatross species were used as case studies in this thesis to test, develop and apply DNA dietary methods to seabird populations. Firstly, the shy albatross (*Thalassarche cauta*) was selected due to its relative accessibility for testing field and laboratory methodologies. Secondly, the circumpolar black-browed albatross (*T. melanophris*) was used to assess the importance of gelatinous prey in albatross diets, and the application of DNA metabarcoding to obtain diet data for ecosystem monitoring and as a fishery resource management tool.

1.6.1 Shy albatross

Shy albatross are endemic to Australia and breed on three islands in Tasmanian waters (Figure 1.1). After a population decline in the 1800s due to exploitation for feathers and eggs, the population has recovered significantly, however their current population is only half that of historic estimates (Alderman et al. 2011). They are currently classified by IUCN as near threatened (IUCN 2015, Table 1.1). The shy albatross colony at Albatross Island in western Bass Strait is the subject of a long-term monitoring study instituted and managed by the Department of Primary Industries, Parks, Water and Environment, Tasmania. The island is relatively accessible compared to other albatross colonies, with three field trips scheduled each breeding season. This colony was therefore ideal to develop, test and optimise field methodologies.

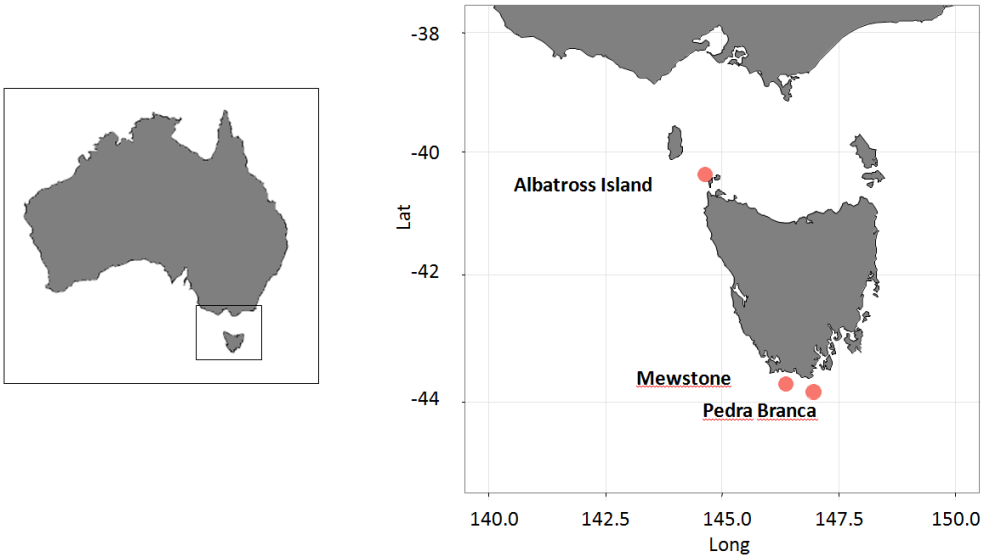

Figure 1.1 Distribution of shy albatross breeding colonies. Sampling occurred at Albatross Island.

1.6.2 Black-browed albatross

The black-browed albatross is a broad-ranging, circumpolar species, which breeds on fourteen islands, or island groups between 45 – 60°S latitude (Figure 1.2). Despite being one of the more numerous albatross species, they have suffered substantial population decline, due primarily to interactions with commercial fisheries (Phillips et al. 2016). Although populations are recovering in some areas (Wolfaardt 2013, Robertson et al. 2014, Robertson et al. 2017), several are still declining (Poncet et al. 2017). Black-browed albatross are currently classified as near threatened under IUCN classification and as threatened under many national legislations (e.g. Brazil and Australia; IUCN 2015; Table 1.1).

Diet studies have been carried out at numerous black-browed albatross colonies (Prince 1980, Reid et al. 1996, Cherel et al. 2000b, Arata and Xavier 2003, Xavier et al. 2003a); however, there has yet to be a coordinated research effort that investigates diet from multiple sites simultaneously or which covers a large proportion of the species range, therefore it is difficult to identify if feeding patterns and behaviours, such as interactions with fisheries, are localised or wide-spread. Black-browed albatross are one of the eight species used in the CCAMLR Ecosystem Monitoring Program to monitor environmental changes and estimate prey consumption. The combination of this and the ongoing interactions with commercial fisheries makes black-browed albatross an ideal species to

assess the utility of DNA metabarcoding as a dietary tool for ecosystem monitoring and fisheries resource management.

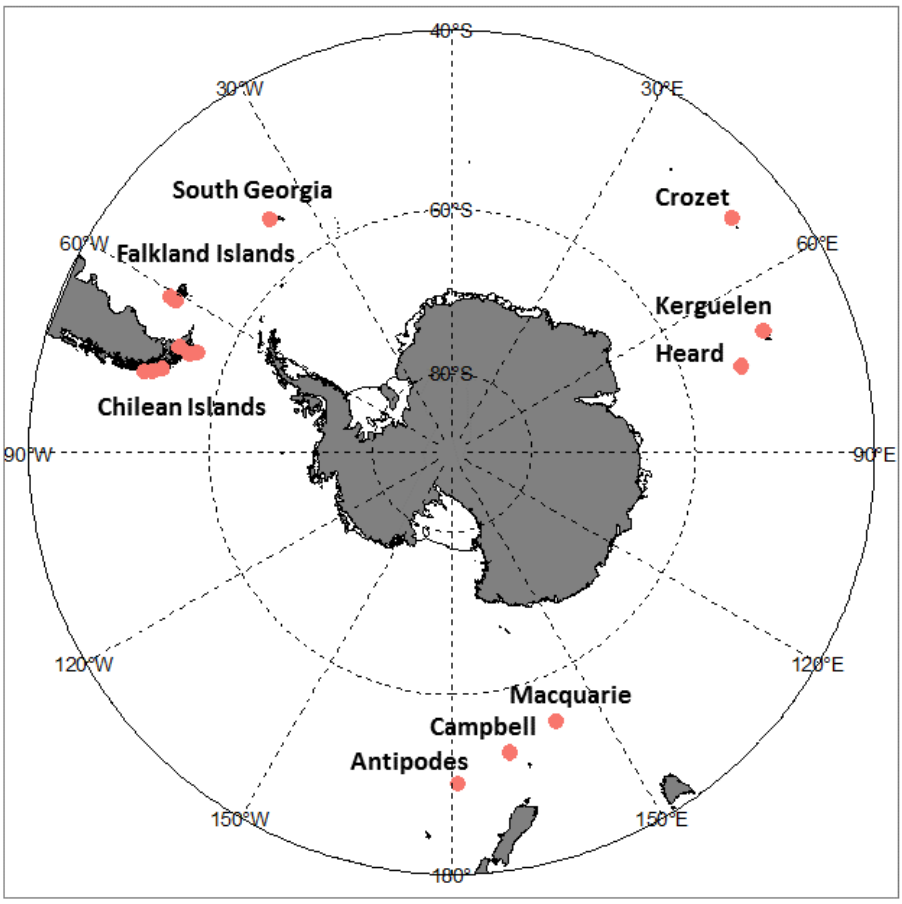

Figure 1.2 Distribution of Black browed albatross breeding colonies

1.7 Thesis structure

This thesis is made up of four chapters that were written as separate manuscripts for publication. As such, there may be some minimal repetition between chapters. All four manuscripts have been published in peer-reviewed journals.

Table 1.1 Albatross Species and population status. Population status is from the International Union for Conservation of Nature (IUCN) Global Red List. Breeding population sizes and distributions are from ACAP Species summaries.

Albatrosses	Scientific name	IUCN Status	Breeding Pairs	Trend	Breeding Colonies
Northern Royal Albatross	*Diomedea sanfordi*	Endangered	5,832	Declining	New Zealand South Island <1%, Chatham Islands >99%
Southern Royal Albatross	*Diomedea epomophora*	Vulnerable	7,886	Stable	Campbell Island (99%), Auckland Islands (1%)
Wandering Albatross	*Diomedea exulans*	Vulnerable	8,050	Declining	Iles Crozet (23%), Iles Kerguelen (15%), Sth Georgia (18%), Prince Edward and Marion Islands (44%), Macquarie Island (<1%)
Antipodean Albatross	*Diomedea antipodensis*	Vulnerable	11,557	Declining	Antipodes Island (46%), Auckland Islands (54%), Campbell Island (<1%)
Amsterdam Albatross	*Diomedea amsterdamensis*	Critically endangered	26	Increasing (ACAP) Declining (IUCN)	Ile Amsterdam (100%)
Tristan Albatross	*Diomedea dabbenena*	Critically endangered	1,763	Declining	Gough Island (>99%), Inaccessible Island (<1%)
Sooty Albatross	*Phoebetria fusca*	Endangered	12,691-14,000	Declining	Prince Edward and Marion Islands (19%), Iles Kerguelen <1%, Iles Crozet 16%, Ile Amsterdam and Ile Saint Paul (4%), Gough and Tristan da Cunha Islands (60%)
Light-mantled Albatross	*Phoebetria palpebrata*	Near threatened	19,261-22,611	Unknown	Heard Island (2%), Macquarie Island (6%), South Georgia (25%), Iles Crozet (11%), Iles Kerguelen (20%), Antipodes Island (1%), Auckland Islands (24%), Campbell Island (8%), Marion and Prince Edward Islands (3%)
Waved Albatross	*Phoebastria irrorata*	Critically endangered	10-15,000	Declining	Galapagos Islands (100%)
Black-footed Albatross	*Phoebastria nigripes*	Near threatened	61,300	Increasing	Hawaiian Islands (95%), Torishima Island (3.5%), Mukojima and Hahajima Rettos (5%)
Laysan Albatross	*Phoebastria immutabilis*	Near threatened	591,356	Stable	Hawaiian Islands (99%), Mexico (East Pacific) (1%)
Short-tailed Albatross	*Phoebastria albatrus*	Vulnerable	470	Increasing	Torishima Island (85%) and Minami-kojima (15%)
Atlantic Yellow-nosed Albatross	*Thalassarche chlororhynchos*	Endangered	26,000-41,000	Declining	Tristan da Cunha group (Tristan, Gough, Nightingale and Inaccessible).
Indian Yellow-nosed Albatross	*Thalassarche carteri*	Endangered	41,000	Declining	Iles Crozet (17%), Iles Kerguelen (50%), Ile Amsterdam (65%), Prince Edward Island (18%)
Grey-headed Albatross	*Thalassarche chrysostoma*	Endangered	95,748	Declining	Macquarie Island (<1%), South Georgia (50%), Iles Crozet (6%), Iles Kerguelen (8%), Diego Ramirez (18%), Campbell (7%), Marion and Prince Edward Islands (11%)
Black-browed Albatross	*Thalassarche melanophris*	Near threatened	~700,000	Variable	Falkland Islands (67%), Macquarie Island (<0.1%), South Georgia (12%), Iles Crozet (0.2%), Iles Kerguelen (0.5%), Diego Ramirez (9.2%), Diego de Almagro (3 %), Islas Ildefonso (8%), Diego Evangelistas (0.8%), Islote Albatros and Islote Leonard (0.1%), Campbell Island and Antipodes Island (<0.1%)
Campbell Albatross	*Thalassarche impavida*	Vulnerable	21,000	Increasing	Campbell Island (100%)
Buller's Albatross	*Thalassarche bulleri*	Near threatened	30,460	Stable	Snares, Solander, Chatham (50%)
Shy Albatross	*Thalassarche cauta*	Near threatened	12,000-15,000	Declining	Tasmania (100%)
White-capped Albatross	*Thalassarche steadi*	Near threatened	97,111	Declining	Auckland Islands 99.9%, Antipodes Island (<0.1%), Chatham Island
Chatham Albatross	*Thalassarche eremita*	Vulnerable	~5,000	Increasing	Chatham Islands (100%)
Salvin's Albatross	*Thalassarche salvini*	Vulnerable	31,974	Unknown	Bounty Island (96%) and Snares Island (4%)

Chapter 2 - A review of methods used to analyse albatross diets – assessing priorities across their range

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"When suddenly alarmed, a bird which has come back from the sea vomits an evil smelling substance, which on examination proved to be a kind of shrimp."

Oliver Austin Jr.
The Status of Steller's Albatross

2.1 Abstract

Many seabird populations are threatened by interactions with commercial fisheries, and climate change. Understanding their prey requirements and dietary flexibility in this context is important for effective conservation and management. However, changes in the methods used to assess diet, as well as the spatial and temporal coverage of monitoring schemes, may reduce our ability to detect and monitor these marine threats. To help assess conservation priorities linked to diet, we carried out a systematic review of 109 albatross diet papers published between 1950 and 2016, which corresponded to 296 studies when stratified by sampling year, breeding site and species. We assessed the methods used, changes over time, and spatial and temporal sampling coverage by species and island group. Most albatross studies have focused on chick-rearing, and diet during other breeding phases is comparatively poorly-known. Furthermore, chicks are more commonly sampled than adults and very rarely immature birds, all of which may differ in diet composition. There was a pronounced shift over time in the preferred method of characterising diet, from the morphological examination of prey remains to stable isotope analysis of tissue. This shift has reduced the volume of detailed taxonomic information available from morphological studies. This difference in resolution hinders the ability to detect changes in prey species, with implications for management of threatened albatrosses and for monitoring broader changes in marine ecosystems. In a knowledge gap analysis for important breeding colonies (with >5% of global population), we identified key sites where existing monitoring has provided a foundation for robust longitudinal diet studies. Maintaining and augmenting these long-term research programmes will enable analyses of the impacts of changing climate and fishing practices on seabird populations, and facilitate the timely identification and implementation of management options.

2.2 Introduction

Two of the greatest marine threats facing seabird populations are interactions with commercial fisheries and global environmental change (Croxall et al. 2002, Chambers et al. 2011). Commercial fisheries often overlap spatially and temporally with seabird foraging areas, and incidental mortality (bycatch) may occur when birds are attracted to vessels to feed on discards or bait (Brothers et al. 1999b). These interactions can be major drivers of population change, and bycatch has been linked to substantial declines in some seabird species (Weimerskirch and Jouventin 1987, Nel et al. 2002a).

Environmental change, primarily driven by anthropogenic carbon emissions, are causing oceans to warm and become more acidic (Feely et al. 2009). These changes affect food-webs and drive spatial and temporal changes in prey abundance and availability, with some prey species predicted to move to cooler waters or alter breeding phenology (Constable et al. 2014). Top predators depend on prey that may be patchily distributed or show seasonal variation. As such, ocean warming may negatively impact predator breeding success if they have insufficient plasticity to adapt to change (Grémillet and Boulinier 2009). Changes in prey availability or distribution can cause prey switching, increase foraging duration, or alter breeding phenology to match seasonal peaks in prey abundance (Gjerdrum et al. 2003, Le Bohec et al. 2008, Xavier et al. 2013). Environmental change may also cause changes to fisheries, including their target species (Barbraud et al. 2012).

Seabirds are highly susceptible to changes in marine ecosystems due to their high trophic position and the predominantly bottom-up control of most food webs (Frederiksen et al. 2006). Changes in prey availability can influence a variety of seabird demographic parameters including breeding success, recruitment and survival. Monitoring of these top predators is therefore important not only for their conservation and management, but also provides indicators of the status of the broader marine ecosystem (Cairns 1987). Collection of dietary data from top predators is an important component of monitoring strategies for many management bodies. For example, in the Southern Ocean, the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) has an Ecosystem Monitoring Program (CEMP) with the principal objective of determining the resource requirements of key top predators, e.g. Adélie penguins (*Pygoscelis adeliae*) and black-browed albatross (*Thalassarche melanophris*, SC-CCAMLR 1997). Similarly, the Southern Ocean Observing System (SOOS) was established to monitor changes in physical and biochemical properties of ocean variables in relation to climate change, including monitoring of albatrosses and other land based predators (Rintoul et al. 2012).

Different types of dietary analysis provide alternative datasets for assessing environmental and fisheries-related changes in marine systems. Seabird diet studies fall into two main categories: those employing morphological analysis to identify remains of prey items from physical attributes, and those using biochemical analyses of tissue samples. Morphological analyses can provide high-level taxonomic identification of prey items, including size estimates. Material for this can be obtained from stomach contents of dead birds by dissection, from live birds by spontaneous regurgitation or stomach lavage, or from pellets (which include indigestible prey remains) or remains of food dropped in the colony (Barrett et al. 2007). Analysis of pellets (also termed boluses) identifies only prey which have indigestible hard parts, primarily cephalopod beaks and fish otoliths, allowing detailed information on species and size class of specific dietary components only (Xavier et al. 2005). Biochemical methods primarily include stable isotope analysis and fatty acids; with techniques such as DNA-based dietary analysis and compound-specific stable isotope in development (Pompanon et al. 2012, Bradley et al. 2014). Stable isotope analysis (SIA) is the main biochemical method used in seabirds and measures ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ (occasionally $^{34}S/^{32}S$) in feathers, blood or other tissues from the target animal, which reflects those in their prey. These measurements integrate the isotopic composition of food consumed during tissue formation, allowing comparisons of habitat use based on environmental gradients in SI ratios, or of trophic level, over periods of days or weeks (Phillips et al. 2009, Cherel et al. 2013). Fatty acid analysis identifies the fatty acid signatures within the adipose tissue of predators and provides information on individual prey groups and species (Budge et al. 2006). Morphological and biochemical analysis convey complementary diet information. Morphological studies identify prey ingested over a short timescale to a high level of taxonomic resolution, which is useful for studying prey selection or consumption of fishery discards. SIA and fatty analysis studies are less labour intensive and provide dietary information derived from prey consumed over a long period of time, which makes them suitable for studying very broad dietary differences at a population scale (Karnovsky et al. 2012).

Albatrosses are one of the most threatened seabird groups, with 16 of 22 species currently on the International Union for Conservation of Nature (IUCN) Global Red List and the other six species classified as Near-threatened (IUCN 2015). Current conservation efforts, coordinated through bodies such as the Agreement for the Conservation of Albatross and Petrels (ACAP) and Birdlife International, are focused on mitigation of known threats to albatross populations, which include fisheries and climate change. Albatross feed on fish, cephalopods, and, in some species, crustaceans and carrion (Cherel and Klages 1998). This paper reviews the methods used to-date to investigate albatross diet and summarises the spatial, taxonomic and temporal coverage of existing studies, with

the aim of identifying gaps in knowledge. Using this threatened group as a case study for other seabirds, we develop a monitoring framework that should allow the detection of dietary responses to changes in the wider environment, including fishing practices, with recommendations for future research and management.

2.3 Methods

We conducted a literature search in February 2016 using Web of Science, Scopus and Google Scholar. Search terms were 'albatross' combined with: 'prey', 'food', 'diet', 'stomach', ' bolus', 'isotope', or 'fatty acid' in any field. Articles were included if they were published in a peer-reviewed journal from 1950 onwards and reported empirical albatross dietary data. Articles were excluded if the title and abstract were not related to albatross or diet analysis, or were based on samples already described in another article. Reference lists of articles, including reviews identified in the literature search, were checked for additional studies.

2.3.1 Dietary database

We extracted the data from each paper for entry into our database as follows: 1) albatross species studied, 2) year of the study, 3) breeding site (island/peninsula), 4) breeding population (island group), 5) stage in annual cycle, 6) sample type, 7) methodology (morphological or biochemical), 8) age class, and 9) sample size (see Table 2.1 for definitions). A study was defined as that in which diet was analysed during a specific breeding or non-breeding season for a species at a site. For example if a paper reported on samples collected from two species over three seasons at one colony, that was considered as six studies. For each study, the conservation status according to the IUCN Red List (IUCN 2015) of each species was included and the approximate population size at that breeding site was derived from published papers, ACAP species assessments (http://www.acap.aq/en/resources/acap-species2) and the Birdlife International online database (http://www.birdlife.org/datazone/species/search).

2.3.2 Synthesis

We reviewed the dataset for spatial and taxonomic gaps in diet sampling, focussing on breeding sites (an individual island or peninsula) that are known to hold > 5% of the global population (ACAP 2015), hereafter termed "IBS" (Important Breeding Sites), and for breeding populations (island groups or mainland areas) that held > 5% of the breeding population, hereafter termed "IBPs" (Important Breeding Populations). Key dietary monitoring sites (KDMS) for each species were identified to

enable ongoing monitoring for the detection of changes over time. Sites were selected where there had been full taxonomic coverage of prey in morphological studies of at least two sets of diet samples.

Table 2.1 Parameters used to categorise diet studies included within the database.

Category	Definition
Scientific name	Based on the 22 species recognised currently by ACAP, BirdLife International and IUCN.
Common name	
Analysis year	The nonbreeding or breeding period that the samples represent. Austral summers were categorised according to the year in which the chicks fledge (e.g. the 2002/2003 season was recorded as 2003). Feathers collected from adults at colonies were assumed to represent the preceding nonbreeding period.
Breeding site	An island with breeding pairs, or in a few cases, a peninsula/clearly disjunct piece of land on the same island.
Breeding population	An island group with pairs breeding, may contain numerous breeding sites
Stage (in annual cycle)	incubation, brood-guard, chick-rearing or nonbreeding period
Sample type	stomach contents (regurgitation, stomach lavage, whole stomach), pellet/bolus/cast, stable isotope (SIA), fatty acid (FAA)
Methodology	morphological, biochemical
Age class	adult, chick or juvenile
Sample size	number of samples analysed (studies with $n \leq 3$ were excluded from analysis). A sample was defined as either a bolus, blood collection, feather or stomach content taken from an individual bird.
IUCN Status	IUCN conservation status, February 2016.
Population size	number of pairs at the breeding site (ACAP 2015).
Reference	

2.4 Results

2.4.1 Search results

Of the 828 papers identified during the literature search, 109 quantified albatross diet in sufficient detail to meet the selection criteria. Of these, 18 papers reported on samples described in more detail elsewhere, therefore the final database included 91 original papers (Appendix 2.1). When stratified by sampling year, breeding site and species, these 85 papers reported on 306 diet studies. Ten were excluded from subsequent analysis as they involved ≤ 3 samples. One study conducted at sea was included even though the sample size was low, because it was the only study for that species. These samples were pooled together as one study (7 samples over 6 years). The 296 studies in the final synthesis were conducted between the late 1940s and 2012 and published between 1950 and 2016.

2.4.2 Dietary analysis techniques

Overall, 65.9% (n=195) of studies used morphological techniques to identify prey, 33.1% used biochemical techniques (n=98), and 1.0% (n=3) involved both techniques. Specifically, the studies were: morphological analyses of stomach contents obtained as regurgitates (45.6%), from dead birds (6.1%), by stomach flushing/lavage (3.4%) or using an unspecified method (2.7%); pellets collected from around nest sites, usually regurgitated by chicks shortly before fledging (17.6%), and, biochemical analyses either of stable isotope ratios (34.1%) or of fatty acids (0.3%). The majority of all studies used one method; however, 9.1% of studies (n=27) used a combination, mostly morphological analyses of regurgitates in combination with either pellets (n=12), stomach contents of dead birds (n=5) or stomach flushing (n=5).

2.4.3 Temporal span

The use of morphological diet studies peaked in the 1970s with an average of 7.0 studies per year. By the 2000s, the number of studies reduced substantially to an average of 1.3 per year from 2001-2012. The first biochemical study of albatross samples using SIA was published in 1997 (on samples collected in 1991), and the first (only) study involving fatty acids was published in 2010 (on samples collected in 2006); the use of SI studies remains high, with an average of 8.8 per year from 2001-2012 (Figure 2.1).

Most studies collected samples during chick-rearing (67.9%), some during the non-breeding period (23.1 %) and more rarely, during incubation (1.0 %) and brood guard (2.2%). In 5.7% of studies, the

breeding stage was not specified (Figure 2.2). Considering all breeding stages, samples were obtained from adults (37.1%), chicks (33.6%), juveniles (0.6%) or multiple age classes (14.9%). The majority of combined studies comprised adults and chicks (13%), four studies of adults and juveniles (1.6%), and two of adults, chicks and juveniles (0.6%). The ages of birds sampled were un-specified in 14.0% of studies (Fig. 2). Biochemical techniques (almost exclusively SIA) were used more often in adults (77.4%) than chicks (21.5%), and rarely in juveniles (2.9%), whereas morphological techniques were more frequent for chicks (58.2%) than adults (38.9%) and again, rarely in juveniles (2.3%; Figure 2.2). Samples from both adults and chicks were collected for the same time period in 42 studies; however, only seven studies compared results from the two age classes.

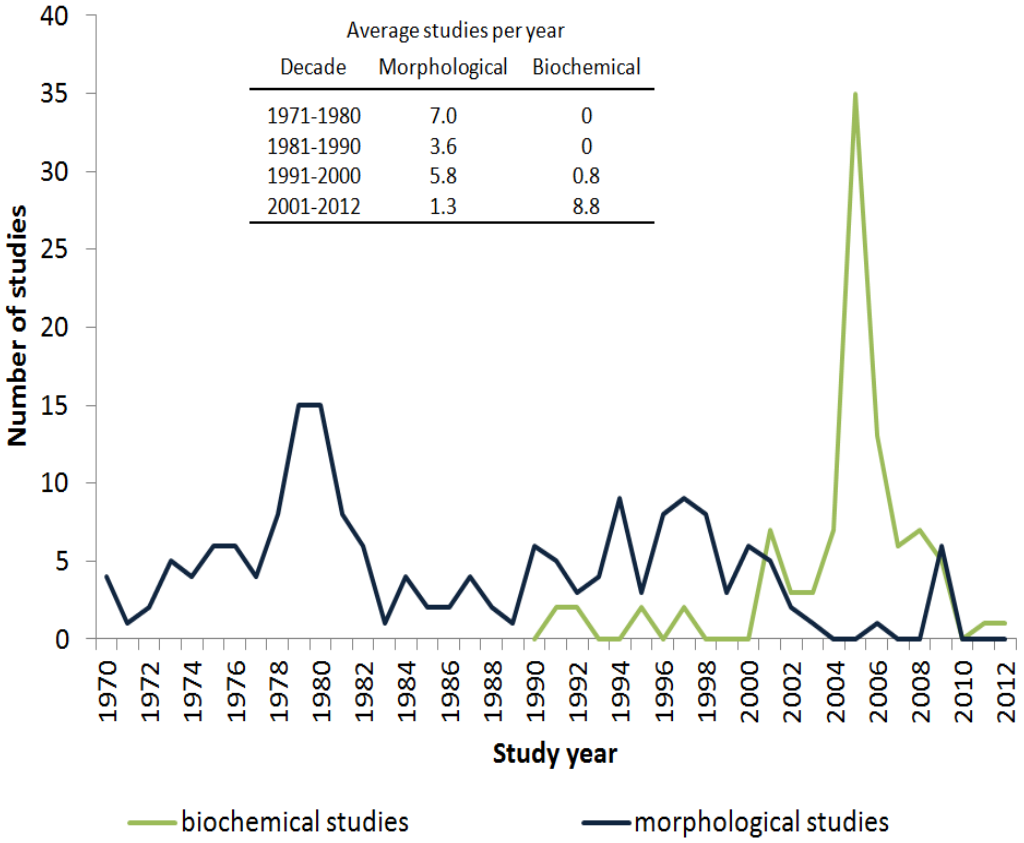
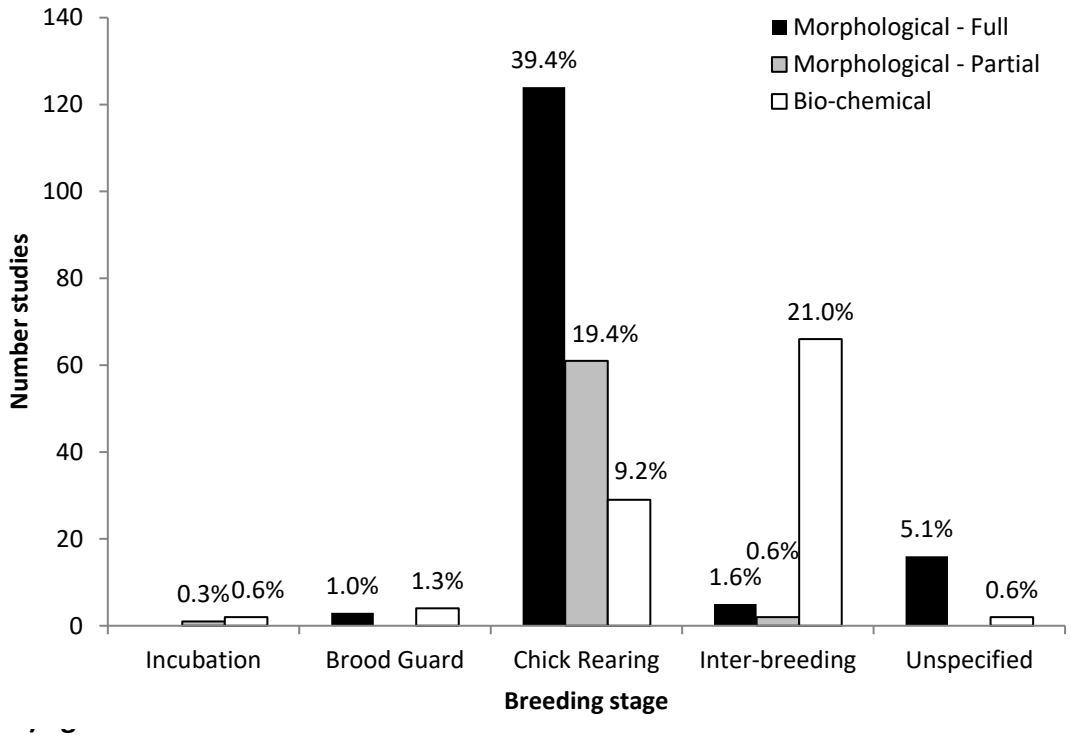


Figure 2.1 The number of diet studies of albatrosses over time using different methods. Morphological - prey identified from physical characteristics (including analysis of regurgitations, stomach contents and pellets). Biochemical - predominantly stable isotope analysis, with one fatty acid study. Ten morphological studies and five biochemical studies were excluded from this graph as the study year was unspecified (Nine morphological studies excluded were published before 1999, and one in 2015).

a) dietary method



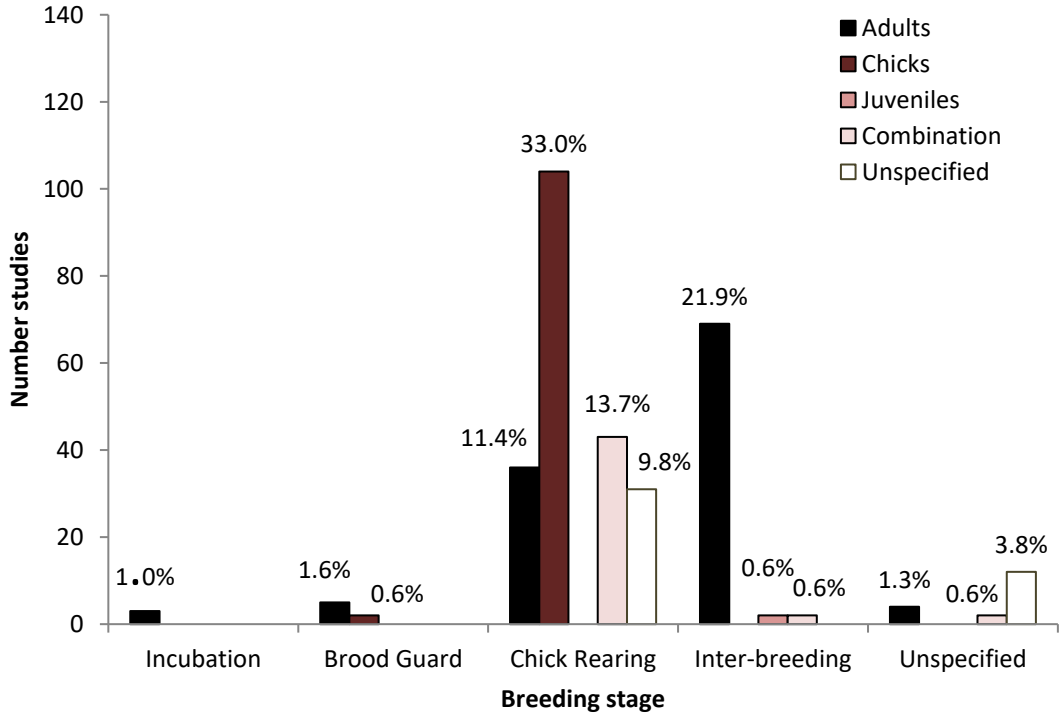


Figure 2.2 The number of albatross diet studies conducted during each major breeding phase, grouped by (a) investigation method (i.e. morphological or bio-chemical analysis) and (b) age class of sampled birds. Each morphological study was categorised as either full or partial, depending on whether multiple prey groups or a single prey group (e.g. cephalopods or fish) were identified.

2.4.4 Species representation

The diet of all 22 currently recognised albatross species has been studied on at least one occasion (Table 2.2). However, morphological data on prey are entirely lacking for Amsterdam (*Diomedea amsterdamensis)*, Chatham (*Thalassarche eremita*), Salvin's (*T. salvini*), and white-capped (*T. steadi*) albatrosses, and are limited for short-tailed albatross (*Phoebastria albatrus*; only 7 samples over 6 years) and Tristan albatross (*D. Dabbenena* -pellets only). Of the 18 species with morphological diet data available, only in six species were these samples collected within the last 10 years (Table 2.2).

2.4.5 Spatial coverage

Diet analyses have been carried-out at 38 breeding locations, corresponding to 65 separate albatross species-site combinations (Figure 2.3). However, many of these studies were at breeding sites that held relatively few birds (Appendix 2.2). There were 67 IBS for which we are able to quantify sampling coverage. A further three island groups are estimated to hold > 5% of the global population of light-mantled albatross (*Phoebetria palpebrata*), but lack reliable counts for the individual islands. Overall, dietary studies have been carried out at 58.2% of IBS (n=39). These include morphological studies at 50.7% of sites (n=34), although only 8.9% (n=6) in the last ten years, and biochemical studies (mostly SIA) at 37.3% of sites (n=25), all but two in the last ten years. There has been at least one diet study at all of the IBS of nine species, less than half the IBS of four species, and at neither of the two IBS of one species (short-tailed albatross) (Table 2.2). Including those for light-mantled albatross (see above), there are 49 important albatross breeding populations (island groups with separate species-site combinations that hold > 5% of the population). There has been at least one diet study on 91.8% of these important breeding populations (n=45); 75.5% have had morphological studies (n=37) and 61.2% have had biochemical studies (n=30).

There have been two full morphological studies of diet for 13 species across 16 sites (28 species/site combinations), and more than two such studies for 8 species across 9 sites (16 species/site combinations), only a minority of which (for four species across five breeding sites; 9 species/site combinations) included at least two sets of samples collected in the last 35 years. These sites are proposed as key dietary monitoring sites (Table 2.3, Figure 2.3).

Table 2.2 Gap analysis of dietary studies using morphological and biochemical approaches for each albatross species. IBS - important breeding sites (an island or peninsula with >5% of the population), IBP - important breeding populations (an island group with >5% of the population). The latest study for each technique is the most recent year a study was carried out.

Species	IUCN Status [a]	Latest morphological Study (year)	No. Morphological studies	Latest biochemical study (year)	No. Biochemical studies	No. IBS	No. IBS with Morphological data	No. IBS with Biochemical data	% IBS with Morphological data	% IBS with Biochemical data	No. IBP	No. IBP with Morphological data	No. IBP with Biochemical data	% IBP with Morphological data	% IBP with Biochemical data
Amsterdam (*Diomedea amsterdamensis*)	CR		0	2007	2	1	0	1	0	100	1	0	1	0	100
Antipodean (*Diomedea antipodensis*)	VU	2001	4	2004	2	2	2	2	100	100	2	2	2	100	100
Atlantic yellow-nosed (*Thalassarche chlororhynchos*)	EN	2004 [d]	2	2007	5	3	1	1	33	33	2	1	1	50	50
Black-browed (*Thalassarche melanophris*)	NT	2009	30 [e]	2012	21 [e]	4	2	1	50	25	4	3	2	75	50
Black-footed (*Phoebastria nigripes*)	NT	1991	13	2006	4	4	3	0	75	0	1	1	0	100	0
Buller's (*Thalassarche bulleri*)	NT	1997	7	2009	2	3	2	1	67	33	3	3	2	100	67
Campbell (*Thalassarche impavida*)	VU	1997	1	1997	1	1	1	1	100	100	1	1	1	100	100
Chatham (*Thalassarche eremita*)	VU	-	0	2008	1	1	0	1	0	100	1	0	1	0	100
Grey-headed (*Thalassarche chrysostoma*)	EN	2009	35 [e]	2005	8 [e]	9	5	3	56	33	6	6	3	100	50
Indian yellow-nosed (*Thalassarche carteri*)	EN	2001	8	2008	3	3	3	2	100	67	3	3	2	100	67
Laysan (*Phoebastria immutabilis*)	NT	2000	14	2007	7	2	2	1	100	50	1	1	0	100	100
Light-mantled (*Phoebetria palpebrata*)	NT	2009	13	2007	9	4 [b]	3	1	75	25	7 [c]	5	3	63	38
Northern royal (*Diomedea sanfordi*)	EN	1993	10	2009	1	3	1	0	33	0	1	1	0	100	0
Salvin's (*Thalassarche salvini*)	VU	-	0	2008	2	8	0	1	0	13	1	0	1	0	100
Short-tailed (*Phoebastria albatrus*)	VU	2014 [d]	1	2006	2	2	0	0	0	0	2	0	0	0	0

Shy (*Thalassarche cauta*)	NT	1998	5	2009	1	2	1	1	50	50	1	1	1	100	100
Sooty (*Phoebetria fusca*)	EN	2009	9	2006	5	5	2	2	40	40	4	3	3	75	75
Southern royal (*Diomedea epomophora*)	VU	1996	7	2004	1	1	1	0	100	0	1	1	0	100	0
Tristan (*Diomedea dabbenena*)	CR	1979	1	2006	3	1	1	1	100	100	1	1	1	100	100
Wandering (*Diomedea exulans*)	VU	2009	36 [e]	2009	19 [e]	5	3	3	60	60	4	3	4	75	100
Waved (*Phoebastria irrorata*)	CR	1971	2	2004	1	1	1	1	100	100	1	1	1	100	100
White-capped (*Thalassarche steadi*)	NT	-	0	2008	1	2	0	1	0	50	1	0	1	0	100
Overall			198 [e]		101 [e]	67	34	25	51%	37%	49	37	30	75%	61%

[a] CR- Critically Endangered; EN- Endangered; VU- Vulnerable; NT- Near Threatened

[b] population count data is unavailable for IBS for some island groups, therefore there may be more IBS for this species that hold >5% of the population

[c] includes three island groups where the individual IBS counts are unavailable, however the total island counts are >5% of the population

[d] samples collected at-sea over numerous years and pooled together for analysis, this is the final year of collection, however only one sample per year.

[e] Three studies have used a combination of both morphological and biochemical methods and are included in both columns.

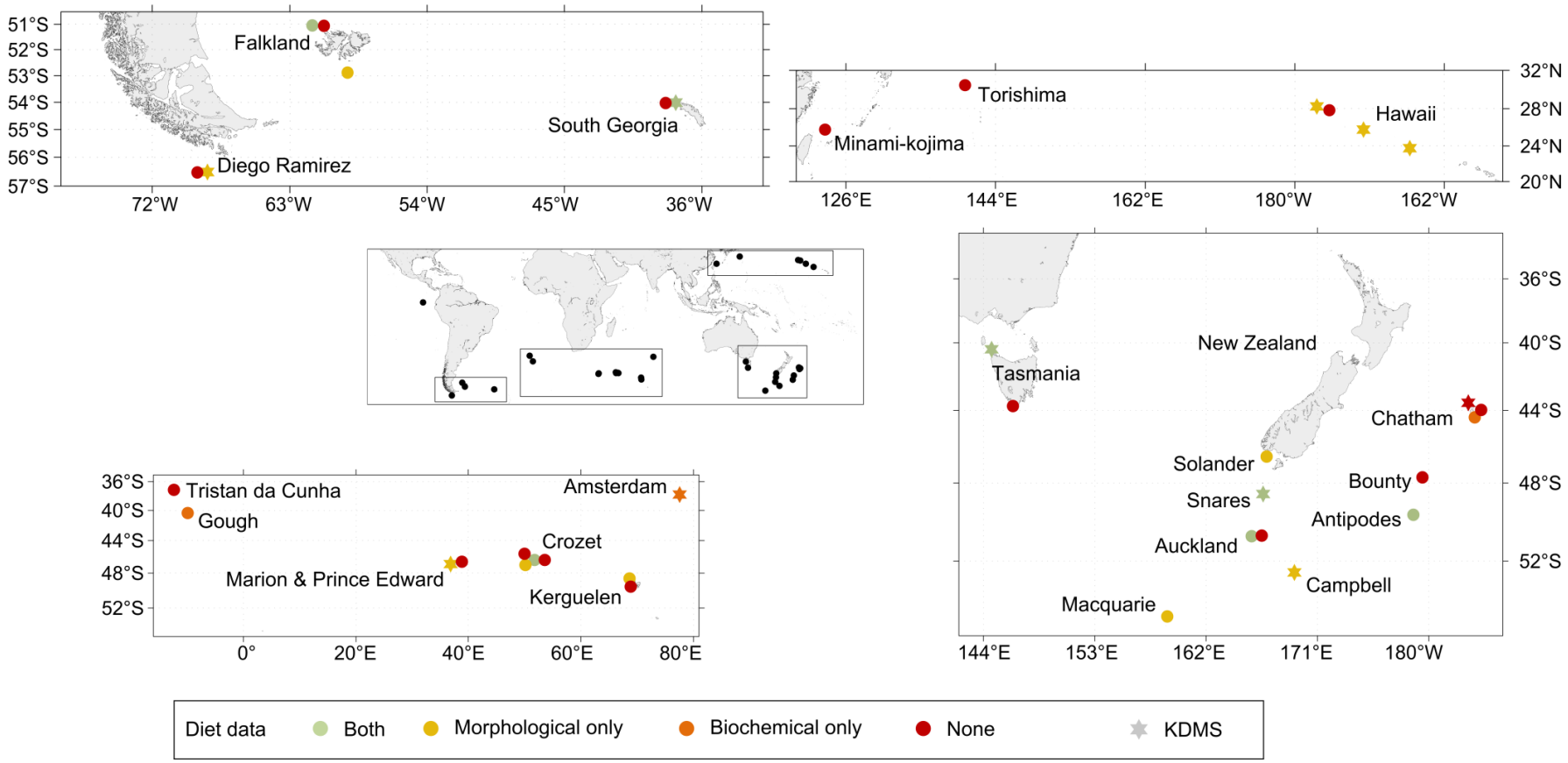


Figure 2.3 Gaps in albatross dietary information at important breeding sites (sites with >5% of the population) and key dietary monitoring sites for ongoing monitoring. Coloured points represent the species at the site with the least dietary information. A star represents a key dietary monitoring site (KDMS), where diet information of high taxonomic resolution has been collected and ongoing monitoring to detect change would be highly beneficial. Not shown in detail on the figure is Isla Espanola in the Galapagos, which is a key dietary monitoring site for waved albatross with both biochemical and morphological dietary information.

Table 2.3 Key dietary monitoring sites. These sites have had at least two full morphological diet studies carried out.

Breeding Site	Breeding Population	Species
Albatross Island	Tasmania	Shy [c]
Bird Island	South Georgia	Black-browed[a,c]
		Grey-headed [c]
		Wandering [c]
Campbell Island	Campbell Island	Grey-headed
Falaise d'Entrecasteaux	Amsterdam Island	Indian yellow-nosed
French Frigate Shoals	Hawaii	Black-footed[b]
		Laysan[a]
Isla Gonzalo	Diego Ramírez	Grey-headed [c]
		Black-browed[a ,c]
Île de la Possession	Crozet Island	Sooty[a]
		Wandering[a, c]
Isla Espanola	Galapagos Islands	Waved [b]
Jeanne d'Arc Peninsula	Kerguelen	Black-browed[a]
Kure Atoll	Hawaii	Black-footed[b]
		Laysan[b]
Laysan Island	Hawaii	Black-footed[b]
		Laysan[b]
Marion Island	Prince Edward Islands	Grey-headed [c]
		Light-mantled[a]
		Sooty
		Wandering [c]
Midway Atoll	Hawaii	Black-footed[b]
		Laysan[b]
North-East Island	Snares Island	Buller's
Taiaroa Head	New Zealand	Northern royal[a]
The Little (Middle) Sister	Chatham Islands	Northern royal
		Buller's[a]

[a] Contain less than 5% of the breeding population.
[b] Most recent morphological diet study > 35 years ago.
[c] Species/sites with *greater* than two full morphological diet studies < 35 years ago.

2.5 Discussion

2.5.1 Species, spatial and temporal dietary information gaps

Many dietary studies of albatrosses have been carried out over the last 40 years, but major gaps still exist in terms of taxonomic, spatial and temporal coverage. The diets of five albatross species have never been studied using morphological methods, or the methods applied provided data with low taxonomic resolution. In addition, only seven samples have been collected over six years from the short-tailed albatross. There are no data on the prey of the critically endangered Amsterdam

albatross, and although this gap is recognised in the recovery plan for this species, sampling of stomach contents by induced regurgitation or stomach lavage is currently considered too invasive (Delord et al. 2011). Other species with no prey information breed in remote locations without permanent research bases and are difficult to access for logistical or political reasons. This includes the Salvin's and white-capped albatrosses breeding on the Bounty and Disappointment islands respectively, Chatham albatross on the privately-owned Pyramid Rock (Robertson et al. 2003), and short-tailed albatross on volcanically-active Torishima Island and the disputed Minami-kojima islands (Appendix 2.3).

The majority of published studies are restricted to the chick-rearing period, largely because chick regurgitations can be collected more easily, less invasively and with less impact than those of adults (Phillips 2006). This has resulted in a major bias in our dietary knowledge towards a single age class and breeding stage. The diet and distribution of adults, and their energy requirements, are known to change with breeding stage (Prince et al. 1994, Bevan et al. 1995). In some species, the availability of specific prey resources at certain times may be critical for successful breeding (Arata and Xavier 2003), and extended foraging trip durations in poor food years can lead to reduced breeding success (Fernandez et al. 2001). Only two studies have investigated diet during incubation, and eight during brood guard, yet in one of the few comparisons between breeding stages, there was a clear seasonal change in prey species composition (Hedd and Gales 2001). Breeding success, and the decision whether or not to breed by albatrosses, have been linked to environmental processes, with potential carryover effects between the breeding and non-breeding seasons (Cuthbert et al. 2003, Rolland et al. 2010). Long-lived species such as albatrosses are expected to prioritise their own survival over that of their offspring when resources are limited, and hence adults that experience poor foraging success are likely to abandon breeding rather than jeopardise their future reproductive potential (Williams 1966). Hence, improved knowledge of how fluctuations in availability of particular prey influence the distribution, breeding success and survival of albatrosses is required to better understand the impacts of ongoing and widespread changes in the marine environment.

The last diet review for albatrosses, published >15 years ago, highlighted the paucity of diet data for the non-breeding period (Cherel and Klages 1998). Since then, SIA has greatly increased our knowledge of trophic level and habitat use during this period, with data now published for 18 species (Phillips et al. 2009, Cherel et al. 2013, Jaeger et al. 2013). Fisheries activity that poses a threat for survival occurs throughout the year, so there remains a conservation imperative for detailed dietary information during the non-breeding season, particularly morphological studies

which provide the most reliable indication of reliance on discards. The difficulty is that most albatrosses do not return to land during the non-breeding period, and although samples can be obtained from birds obtained as fisheries bycatch (Gould et al. 1997, Colabuono and Vooren 2007), these will be biased to ship-following individuals. An alternative is to obtain samples from adults that have returned to colonies (Alvito et al. 2015), and potentially use SI ratios in feathers, natural prey and discards, to distinguish the fisheries contribution to the diet using stable isotope mixing models (Bugoni et al. 2010, Granadeiro et al. 2013). Understanding the diet and distribution of juveniles is also important, as survival is at its lowest between fledging and returning to breed (Terauds et al. 2006b, Alderman et al. 2010). To date, only one study has presented a morphological diet breakdown for juveniles (Colabuono and Vooren 2007), and although samples from juveniles were collected in five other studies, these were pooled with other age classes (West and Imber 1986). Comparison of juvenile and adult foraging ecology in wandering albatrosses was recently carried out using SIA (Jaeger et al. 2014). Juveniles and non-breeding birds fed in sub-tropical waters, similar to breeding females, with males switching to colder waters during breeding. This study provides an important insight into juvenile behaviour and the different life history strategies of different age, sex and breeding classes that lead to diet variation.

Our analysis of the spatial and taxonomic coverage of dietary studies identified 28 IBS that are data-deficient. For many of these important colonies, there is no information on diet composition, including the largest breeding populations of grey-headed, shy, Buller's and northern royal albatrosses. In some cases, there are data for another breeding site in close proximity. For example, diets of grey-headed albatross at the IBS at the Paryadin Peninsula (South Georgia) and Isla Bartolome (Islas Diego Ramirez) are unknown, but there have been studies at Bird Island and Isla Gonzalo, respectively, which are within the same island groups. It is difficult to quantify the spatial scale at which diet variation occurs, given many confounding effects such as breeding stage, season, age, sex and resource availability. Diet can differ considerably between islands within relatively close proximity (Thompson 1992); thus, any breeding site or island group that holds a substantial proportion of the global population is potentially an important target for a diet study, as is any site considered to be a conservation or management priority. By using tracking studies and SIA, it would be useful to identify IBS where birds have substantially different foraging distributions or feed at distinct trophic levels. The former could be distinguished using the Seabird Tracking Database of Birdlife International (http://www.seabirdtracking.org/index.php), and the latter using existing SI data.

2.5.2 Diet analyses

Each method of dietary analysis has advantages and disadvantages (for reviews see Barrett et al. 2007, Karnovsky et al. 2012). There is no single method that identifies all the ages, size classes and species of prey that an animal ingests. Some seabird studies have tried to achieve this by incorporating a variety of methods and models (e.g. Chiaradia et al. 2014), yet it remains difficult to characterise the true diet of top predators. The relative importance of certain prey in stomach contents can be biased by differential rates of digestion, leading to under-estimation of soft-bodied prey and over-representation of prey with hard parts, particularly squid, that tend to be retained for long periods (Furness et al. 1984). More recent studies tend to analyse fresh and old squid beaks separately to compare species composition over the short- or long-term (Cherel et al. 2000b); however, underestimation of soft-bodied prey is still an issue. Pellets only include prey that have indigestible hard parts, primarily squid (Xavier et al. 2005), and large beaks (from large species and individuals) are often over-represented, as small beaks tend to degrade (Brooke and Klages 1986). Stable isotope and fatty acid analysis does not suffer from the same issue of prey retention and provides a less biased view of prey components. Rodhouse et al. (2013), reviewed a number of studies where SIA and fatty acids have highlighted the importance of fish prey for predators that had been assumed to eat mainly squid due to the retention of beaks in stomachs. Although these biochemical techniques avoid biases from prey retention, taxonomic resolution is typically very poor because SI ratios and lipid information for many prey species are unavailable, or overlapping (Inger and Bearhop 2008).

DNA-based approaches to diet analysis and compound-specific SIA are new techniques that may allow some of the knowledge gaps identified in this review to be filled, potentially overcoming some of the limitations of stomach contents analysis. DNA-based dietary analysis is a non-invasive means of identifying individual prey species in the diet by detecting the genetic sequences in the predator's faeces (Pompanon et al. 2012, Bowser et al. 2013, Jarman et al. 2013). This method can be used during all breeding and life-history stages when scats can be collected (Bowser et al. 2013, McInnes et al. 2016a). As no handling of birds is required, it provides an ideal method of assessing the diet of sensitive species. The limitations are that prey age, size class and mass cannot be assessed. Scats must also be available, so the approach is not feasible for determining diet during the non-breeding period for species that remain far from land. Compound-specific SIA methods have the potential to provide more detailed taxonomic identifications than standard SIA, but these methods are new and their utility is still being explored (Bradley et al. 2014).

To determine the most suitable dietary method is a balance between method limitations, disturbance and the information required (Table 2.4). To identify prey specific overlaps between albatrosses and fisheries, analysis of stomach contents is ideal as the majority of target prey (fish and squid) have identifiable hard-parts (Arata and Xavier 2003). However, fish prey can still be difficult to identify when no hard parts remain (e.g.Ridoux 1994). In contrast, SIA is also very useful for identifying reliance on fisheries waste, particularly where discards from demersal or pelagic fisheries differ isotopically from natural prey (Bugoni et al. 2010). When identifying changes in diet over time due to environmental conditions, SIA and fatty acids can again highlight broad trophic changes, but would ideally be complemented with information on all prey components. Although pellets and stomach contents can identify changes in cephalopod and some other prey components, no dietary method currently used for albatrosses can reliably quantify the contribution of soft-bodied prey. This information will be important as the abundance of gelatinous prey is likely to increase with warming oceans (Attrill et al. 2007). DNA-based dietary analysis may be the ideal method to complement SIA in the future to enable detection of soft-bodied prey (McInnes et al. 2016a); however, hard-part analysis will be required if changes in prey size classes are to be investigated. Miniaturized cameras or video loggers are now small enough to be used on seabirds and allow another platform for observing prey selection and overlaps with fisheries (Votier et al. 2013). These cameras can detect soft-bodied prey (Thiebot et al. 2016) and could be used to understand the importance of this prey group for albatross.

2.5.3 A synoptic strategy for albatross dietary studies

This review provides a framework for prioritising global monitoring of albatross diets. To enable the detection of prey changes resulting from climate change or fisheries practices, two key improvements are necessary: 1) an increase in the collection of taxonomically-detailed prey information and 2) maintenance and expansion of the network of long-term monitoring sites.

Increase the collection of prey information

Comprehensive morphological studies are either entirely lacking, or have not been conducted in the last 35 years, for one third of albatross species, including three Critically Endangered species (Table 2.2). Furthermore, in the last decade, there has been a morphological study of the diet of only six species. This general trend away from morphological techniques may reflect the more labour-intensive nature of identifying individual prey and lack of sufficient taxonomic skills, or concerns about biases associated with greater retention of hard parts and faster digestion of soft-bodied prey (Barrett et al. 2007). The number of diet studies involving stomach contents has also declined,

possibly because of the perceived detrimental effects on chick survival. Although sampling in this way had minimal impact on chick condition or breeding success (Phillips 2006), the associated disturbance and loss of a meal may be discouraging its wider use. Indeed, the management plans for several albatross species state specifically that diet information is lacking due to the invasive nature of current techniques (Delord et al. 2011, DSEWPAC 2011).

The observed shift away from morphological analysis of diet has reduced our ability to detect all but major prey shifts, and yet has occurred against a background of major perturbations in marine ecosystems, driven by a range of climate-related processes (Constable et al. 2014). These will continue to influence the availability of food resources for albatrosses and their prey (Hays et al. 2005). Environmental changes also alter the operation of fisheries, either by a decline or shift in distribution of target stocks. Monitoring the impacts of these changes on albatross diet would ideally involve complementary approaches: SIA to identify broad shifts in diet, and morphological analyses to provide taxonomic detail to address more targeted questions, such as the effects of changes in distribution or abundance of particular prey, or proposed changes in fisheries practices. In time, it is hoped the development of new methods e.g. DNA-based dietary analysis, may help fill this gap in taxonomic resolution, but in the meantime, resumed collection of morphological data is important to detect any prey changes.

Maintain and expand the network of long-term monitoring sites

Ongoing monitoring of key sites where there are existing time-series of robust diet data is imperative for detecting dietary shifts and identifying links between prey abundance and albatross demography. Long-term diet data is invaluable for enabling management and protection of species and their prey resources, and for observing changes to the wider marine system. The CCAMLR Ecosystem Monitoring Program (CEMP; SC-CCAMLR 1997) uses top predators such as the black-browed albatross as indicator species to monitor change in the marine environment, particularly in relation to competition with the fishery for Antarctic krill (*Euphausia superba*), and requires predator diet information in order to estimate consumption. The ongoing CEMP diet sampling programme of black-browed albatross at Bird Island (South Georgia) has provided one of the longest diet time-series for any site (>15 years; Appendix 2.3). However, there are very few long-term dietary projects for albatrosses; only at five sites are there more than two full dietary studies published in the last 35 years. This makes it difficult to detect diet shifts, and even harder to attribute these to fishing activity or other changes in the environment. For these reasons, the implementation of a wider diet monitoring framework for albatrosses should be encouraged by international agreements such as

ACAP and CCAMLR, or NGOs, including BirdLife International, and incorporated into national plans of action and recovery plans.

The proposed KDMS are a recommended starting point for establishing long-term monitoring sites as they allow comparison with existing dietary data. However, they should not be interpreted as the only sites, as there may well be others that are biogeographic, conservation or other management priorities. This network of sites identified here should be refined in the future, by using fisheries, environmental and tracking data to determine the pressures that affect different IBSs and IBPs.

Further linkages between diet studies and other research avenues should be strengthened. Several studies have linked diet composition to foraging location using tracking data (e.g.Xavier et al. 2003c), and to breeding success (e.g.Arata et al. 2004). Further work integrating longitudinal tracking and diet studies would allow assessment of changes in important foraging areas or food resources, and how this may affect breeding success. This will be important to identify projected environmental changes and how species may adapt under different climate change scenarios or fishing impacts. Furthermore, by linking tracking to diet, we can continue to identify geographic overlaps with fisheries (Xavier et al. 2004) as well as likely location of prey (Xavier et al. 2006)

An open-access database of prey and SI information would assist in establishing distributions of prey species and monitoring spatial and temporal changes. A centralised approach would ensure uniformity in data collection and reporting, and enable collaboration and coordination between management authorities. One such database has been established for the Southern Ocean (Raymond et al. 2011) and at the time of writing, the Expert Group on Birds and Marine Mammals within the Scientific Committee on Antarctic Research, are extending this database into a Southern Ocean community resource with expanded coverage of SI and other diet data (including the data compiled for the current study). Companion efforts would, ideally, broaden the database coverage to include albatross breeding sites elsewhere. An updated dietary database would provide the foundation for a thorough synthesis of prey and SI data, including a meta-analysis identifying the important prey components for each species, and spatial and temporal variation.

Table 2.4 Dietary methods used to assess albatross diet, with requirements, associated biases, disturbance and additional information obtained.

Method	Sample period	Taxonomic Resolution	Prey database required	Relative Disturbance	Key limitations	Unit of Measure	Additional Information
Regurgitation	Days (flesh remains) Weeks (hard-part remains)	Species/group	Prey hard-parts	Medium	Over-estimation of prey with hard parts, under-estimation of soft-bodied prey, provisioning diet only for many species, potentially only partial stomach contents obtained.	FOC, Mass, proportion of items	Prey size, reconstituted meal mass
Stomach flushing	Days (flesh remains) Weeks (hard-part remains)	Species/group	Prey hard-parts	High	Over-estimation of prey with hard parts, under-estimation of soft-bodied prey.	FOO, Mass, proportion of items	Prey size, reconstituted meal mass
Stomach content of dead birds	Days (flesh remains) Weeks (hard-part remains)	Species/group	Prey hard-parts	Low	Over-estimation of prey with hard parts, under-estimation of soft-bodied prey, potentially confounded by cause of mortality.	FOO, Mass, proportion of items	Prey size, reconstituted meal mass
Pellets (boluses)	Weeks (hard-part remains)	Species with hard parts	Prey hard-parts	Low	Only identifies prey with hard-parts.	FOO, Proportion of items	Prey size
SIA	Days (using blood) Months (using feathers)	Trophic level	Prey stable isotope signatures	Medium-High	Low taxonomic resolution (potentially improved using multi-source isotope mixing model).	FOO, relative proportions from mixing models	Foraging habitat (carbon source)
DNA	Days (faeces)	Species/genus and broad diet	Prey genetic sequences	Low	Lacks mass and size information of prey, semi-quantitative.	FOO, proportion of sequences	Bird sex, parasites
Fatty Acids	Days (using blood) Months (using muscle or fat)	Trophic level	Prey fatty acid signatures	High	Low taxonomic resolution.	FOO, relative proportions from mixing models	Foraging habitat

FOO= Frequency of co-occurrence

2.5.4 Conclusion

On the basis of the gaps and trends identified in this review, we have outlined key recommendations for future dietary work (Table 2.5). These are made specifically in the context of albatross conservation management; however, they are likely to be applicable across many other seabird groups. Our work highlights the pressing need for dietary studies both of highly threatened species and major breeding sites in general. Taxonomic, spatial and temporal gaps exist in baseline sampling coverage, particularly with regard to poorly-known stages of the breeding season, the non-breeding period, juveniles and immature birds. The development of new non-invasive techniques with high taxonomic resolution may assist in this process, and will be particularly useful for sensitive species where disturbance is a major concern. Collecting information on seabird dietary requirements is fundamental to enable monitoring of their prey, and should be designed to detect diet shifts over short and long time-scales. Understanding how these prey changes affect breeding and non-breeding birds will be crucial for gauging the threat these changing processes pose to seabird populations. Ongoing diet monitoring across a network of key sites should be seen as a high priority for conservation and management bodies, as should the continued collection of prey information to complement SIA and enable detection of fine-scale differences in abundance and utilisation of key individual taxa. The key findings, recommendations and proposed actions identified in this paper (Table 2.5), will hopefully enable the detection and attribution of the impacts of climate or fisheries processes on albatross populations and facilitate the timely implementation of responsive management.

2.6 Acknowledgements

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Table 2.5 Summary of key findings, recommendations and actions for ongoing albatross dietary monitoring.

Key Findings	Recommendation	Proposed Action
Knowledge gaps Significant gaps in prey information exist for: Amsterdam, Chatham, Salvin's, and white-capped albatross, with limited information for Tristan and short-tailed albatross.	Prioritise filling the gaps for the species and sites where such information is needed to make a difference to conservation and management.	ACAP/Birdlife International to facilitate the prioritisation of species and IBS to investigate diet. Potentially use tracking databases and existing stable isotope and prey data to determine monitoring priorities.
	Increase monitoring to incorporate other stages of the breeding and non-breeding seasons. This may potentially be facilitated by a combination of SIA and the utilisation of new techniques such as DNA dietary analysis.	Incorporate appropriate dietary studies as an integral component of species recovery and management plans. Elevate the importance of dietary studies in long term monitoring plans to link observed demographic parameters to ecological drivers.
Prey information mostly restricted to chick rearing Reduction in resolution of information on prey items consumed due to changes in methodology	Continue to progress the application of DNA and other forensic dietary analyses for albatross. DNA analysis may allow some of these gaps to be filled as it is logistically straightforward to collect and provides detailed prey information. Resume collection of prey information either using morphological examination of hard parts or DNA dietary techniques, to complement SIA.	Undertake trials to check the feasibility of DNA and other forensic methods for albatross.
Detecting change Difficulty in detecting change due to limited long-term data collection.	Maintain long-term research at key sites to enable robust longitudinal diet assessment and maximise the outputs from past investment in such studies.	Use the key dietary monitoring sites (Table 2.3) as a basis for an implementation plan to enable longer time-series data to be collected.
	Compile diet data including prey and SIA at a centralised location to enable detection of changes over time	Develop a centralised top-predator database of diet information to facilitate quantification of changes in prey over temporal and spatial scales. Such a database could include similar information from other marine predators to allow evaluation of impacts of climate and fisheries changes at an ecosystem level. Co-ordination with EGBAMM within SCAR* who have begun a similar database for SI data.
	To achieve consistency in data collection, adopt standardised methods of collecting and reporting dietary data that enables comparisons over time. These should likely be based on existing protocols such as the CCAMLR Ecosystem Monitoring Program Standard Methods (SC-CCAMR 1997).	Work with organisations such as ACAP to encourage signatories to improve diet monitoring. Repeat SIA studies at the same sites to identify any major shifts in prey or habitat use.

*EGBAMM – expert group on birds and marine mammals within the Scientific Committee on Antarctic Research (SCAR)

2.7 Appendices

Appendix 2.1: Published diet studies for the 22 albatross species.

Species	Reference
Amsterdam (*Diomedea amsterdamensis*)	(Cherel et al. 2013, Jaeger et al. 2013)
Antipodean (*Diomedea antipodensis*)	(Imber and Russ 1975, Imber 1992, Cherel et al. 2013, Xavier et al. 2014)
Atlantic Yellow-nosed (*Thalassarche chlororhynchos*)	(Hagen 1952, Colabuono et al. 2007, Bugoni et al. 2010, Cherel et al. 2013, Jaeger et al. 2013, Colabuono et al. 2014)
Black-browed (*Thalassarche melanophris*)	(Tickell 1964, Prince 1980, Clarke and Prince 1981, Weimerskirch et al. 1986, Thompson 1992, Rodhouse and Prince 1993, Ridoux 1994, Cherel and Weimerskirch 1995, Thompson and Riddy 1995, Reid et al. 1996, Rodhouse et al. 1996, Croxall et al. 1997, Croxall et al. 1999, Cherel et al. 2000a, Cherel et al. 2000b, 2002, Arata and Xavier 2003, Xavier et al. 2003a, Xavier et al. 2005, Colabuono et al. 2007, Colabuono and Vooren 2007, Petry et al. 2007, Suazo 2008, Anderson et al. 2009, Phillips et al. 2009, Weiss et al. 2009, Bugoni et al. 2010, Phillips et al. 2011, Cherel et al. 2013, Granadeiro et al. 2013, Jaeger et al. 2013, Colabuono et al. 2014, Mariano-Jelicich et al. 2014, Alvito et al. 2015)
Black-footed (*Phoebastria nigripes*)	(Harrison et al. 1983, Gould et al. 1997, Suryan and Fischer 2010)
Buller's (*Thalassarche bulleri*)	(West and Imber 1986, James and Stahl 2000, Cherel et al. 2013)
Campbell (*Thalassarche impavida*)	(Cherel et al. 1999, Waugh et al. 1999, Cherel et al. 2013)
Chatham (*Thalassarche eremita*)	(Cherel et al. 2013)
Grey-headed (*Thalassarche chrysostoma*)	(Bailey and Sorensen 1962, Tickell 1964, Prince 1980, Tarburton 1980, Clarke and Prince 1981, Brooke and Klages 1986, Weimerskirch et al. 1986, Hunter and Klages 1989, Rodhouse et al. 1990, Ridoux 1994, Reid et al. 1996, Rodhouse et al. 1996, Croxall et al. 1997, Croxall et al. 1999, Waugh et al. 1999, Nel et al. 2000, Nel et al. 2001, Cherel et al. 2002, Xavier et al. 2003a, Xavier et al. 2003c, Arata et al. 2004, Catry et al. 2004, Xavier et al. 2005, Anderson et al. 2009, Phillips et al. 2009, Richoux et al. 2010, Phillips et al. 2011, Cherel et al. 2013, Jaeger et al. 2013, Connan et al. 2014, Alvito et al. 2015)
Indian Yellow-nosed (*Thalassarche carteri*)	(Brooke and Klages 1986, Weimerskirch et al. 1986, Ridoux 1994, Cherel et al. 2002, Pinaud et al. 2005, Cherel et al. 2013, Jaeger et al. 2013)
Laysan (*Phoebastria immutabilis*)	(Harrison et al. 1983, Gould et al. 1997, Pitman et al. 2004, Suryan and Fischer 2010, Edwards et al. 2015)
Light-mantled (*Phoebetria palpebrata*)	(Sorensen 1950, Mougin 1970a, Berruti and Harcus 1978, Thomas 1982, Weimerskirch et al. 1986, Ridoux 1994, Cooper and Klages 1995, Green et al. 1998, Phillips et al. 2009, Jaeger et al. 2010, Cherel et al. 2013, Jaeger et al. 2013, Connan et al. 2014)
Northern Royal (*Diomedea sanfordi*)	(Marchant and Higgins 1990, Imber 1991, Imber 1999, Cherel et al. 2013)
Salvin's (*Thalassarche salvini*)	(Cherel et al. 2013)
Short-tailed (*Phoebastria albatrus*)	(Suryan and Fischer 2010, Walker et al. 2015)
Shy (*Thalassarche cauta*)	(Green 1974, Hedd and Gales 2001, Cherel et al. 2013)

Sooty (*Phoebetria fusca*)	(Mougin 1970a, Berruti and Harcus 1978, Richardson 1984, Weimerskirch et al. 1986, Ridoux 1994, Cooper and Klages 1995, Jaeger et al. 2010, Cherel et al. 2013, Connan et al. 2014)
Southern Royal (*Diomedea epomophora*)	(Marchant and Higgins 1990, Imber 1999, Battley et al. 2008, Cherel et al. 2013)
Tristan (*Diomedea dabbenena*)	(Imber 1992, Bugoni et al. 2010, Cherel et al. 2013, Jaeger et al. 2013, Colabuono et al. 2014)
Wandering (*Diomedea exulans*)	(Voisin 1969, Mougin 1970b, Croxall and Prince 1980, Clarke et al. 1981, Imber and Berruti 1981, Weimerskirch et al. 1986, Prince and Morgan 1987, Rodhouse et al. 1987, Croxall et al. 1988, Cooper et al. 1992, Imber 1992, Weimerskirch et al. 1997a, Cherel and Weimerskirch 1999, van den Hoff 2001, Nel et al. 2002b, Xavier et al. 2002, Xavier et al. 2003b, Xavier et al. 2003c, Xavier et al. 2004, Weimerskirch et al. 2005, Xavier et al. 2006, Xavier and Croxall 2007, Anderson et al. 2009, Jaeger et al. 2009, Phillips et al. 2009, Bugoni et al. 2010, Jaeger et al. 2010, Phillips et al. 2011, Ceia et al. 2012, Cherel et al. 2013, Jaeger et al. 2013, Colabuono et al. 2014, Jaeger et al. 2014, Ceia et al. 2015, Guerreiro et al. 2015, Seco et al. 2015)
Waved (*Phoebastria irrorata*)	(Harris 1973, Awkerman et al. 2007)
White-Capped (*Thalassarche steadi*)	(Cherel et al. 2013)

Additional reviews:

(Croxall and Prince 1994, Croxall and Prince 1996, Cherel and Klages 1998, Xavier and Croxall 2007, Xavier et al. 2007, Vaske 2011, Xavier et al. 2013, Moreno et al. 2016)

Appendix 2.2 Breeding sites with <5% of the global population where the diet has been characterised using one or more approaches. N – No study, C – cephalopods identified, F – fish identified, Cr – crustaceans identified, A – all taxa identified, B – broad prey groups identified without species information, SIA – stable isotope analysis, studies during the non-breeding season have NB in parenthesis.

Common Name	IUCN Status	Breeding Site	Breeding population	Jurisdiction	Annual Breeding pairs	Morphological	Biochemical	Total Studies
Black-browed	NT	Bird Island	South Georgia	UK	8264	A, C(NB)	SIA, SIA (NB)	19
Black-browed	NT	Isla Gonzalo	Diego Ramírez	Chile	6897	A	N	3
Black-browed	NT	New Island	Falklands/Malvinas	UK	13343	A	SIA, SIA (NB)	7
Black-browed	NT	Heard Island	Heard	Australia	600	N	SIA	1
Black-browed	NT	Île de l'Est	Crozet	France	350	B, C, Cr	N	4
Black-browed	NT	Île de Croÿ	Kerguelen	France	1815	A	N	1
Black-browed	NT	Jeanne d'Arc Peninsula	Kerguelen	France	1081	A	SIA, SIA (NB)	5
Black-footed	NT	Kure Atoll	Hawaiian	USA	3434	A	N	2
Buller's	NT	The Little (Middle) Sister	Chatham	NZ	650	A	SIA	3
Grey-headed	EN	Île de l'Est	Crozet	France	3750	B, C, Cr	N	4
Grey-headed	EN	Prince Edward Island	Prince Edward	South Africa	1506	A	N	3
Indian yellow-nosed	EN	Îles Nuageuses	Kerguelen	France	50	A	N	1
Laysan	NT	Kure Atoll	Hawaiian	USA	24366	A	N	2
Laysan	NT	French Frigate Shoals	Hawaii	USA	3063	A	N	3
Laysan	NT	Isla Guadalupe	Guadalupe	Mexico	562	C	N	1
Light-mantled	NT	Heard Island	Heard Island	Australia	350	C	N	1
Light-mantled	NT	Marion Island	Prince Edward	South Africa	316	A	SIA, SIA (NB)	5
Northern royal	EN	Taiaroa Head	New Zealand	NZ	36	A	N	6
Salvin's	NT	Toru Islet	Snares Island	NZ	829	N	SIA	1
Sooty	EN	Île Amsterdam	Amsterdam	France	394	N	SIA	1
Sooty	EN	Île de la Possession	Crozet	France	81	A	SIA, SIA (NB)	7
Southern royal	NT	Adams Island	Auckland	NZ	15	N	SIA	1
Wandering	VU	Île de la Possession	Crozet	France	371	A	SIA, SIA (NB)	14
Wandering	NT	Courbet Peninsula	Kerguelen	France	356	C	SIA, SIA (NB)	3
Wandering	NT	Macquarie Island	Macquarie	Australia	6	C	N	3

Appendix 2.3 List of important breeding sites (with >5% of the global population) where diet has been characterised using one or more approaches and those without diet studies. N – No study, C – cephalopods identified, F – fish identified, Cr – crustaceans identified, A – all taxa identified, B – broad prey groups identified without species information, SIA – stable isotope analysis. Studies during the non-breeding season have NB in parenthesis.

Common name	IUCN Status	Breeding Site	Breeding population	Jurisdiction	Annual Breeding pairs	>10% pop	Morphological	Biochemical	Total Studies
Amsterdam	CR	Plateau des Tourbières	Amsterdam	France	31	Y	N	SIA, SIA (NB)	2
Antipodean	VU	Adams Island	Auckland	NZ	3277	Y	B, F, C	SIA (NB)	3
Antipodean	VU	Antipodes Island	Antipodes	NZ	4565	Y	C	SIA (NB)	3
Atlantic yellow-nosed	EN	Gough Island	Gough	UK	5300	Y	N	SIA, SIA (NB)	2
Atlantic yellow-nosed	EN	Nightingale	Tristan da Cunha	UK	4000	Y	N	N	0
Atlantic yellow-nosed	EN	Tristan da Cunha	Tristan da Cunha	UK	23000	Y	B	N	1
Black-browed	NT	Beauchene Island	Falklands/Malvinas	UK	105777	Y	A	N	1
Black-browed	NT	Grand Jason	Falklands/Malvinas	UK	89489	Y	N	N	0
Black-browed	NT	Isla Bartolomé	Diego Ramírez	Chile	43304	N	N	N	0
Black-browed	NT	Steeple Jason	Falklands/Malvinas	UK	171286	Y	A	SIA	2
Black-footed	NT	French Frigate Shoals	Hawaiian	USA	4944	N	A	N	3
Black-footed	NT	Laysan Island	Hawaiian	USA	24565	Y	A	N	3
Black-footed	NT	Midway Atoll	Hawaiian	USA	27498	Y	A	SIA	4
Black-footed	NT	Pearl and Hermes Reef	Hawaiian	USA	6116	N	N	N	0
Buller's	NT	Great Solander Island	Solander	NZ	4579	Y	A	N	1
Buller's	NT	North-East Island	Snares	NZ	7898	Y	A	SIA (NB)	5
Buller's	NT	The Forty-fours	Chatham	NZ	14185	Y	N	N	0
Campbell	VU	Campbell Island	Campbell	NZ	22093	Y	A	SIA (NB)	2
Chatham	VU	The Pyramid	Chatham	NZ	5245	Y	N	SIA (NB)	1
Grey-headed	EN	Bird Island	South Georgia	UK	5120	N	A, C(NB)	SIA, SIA (NB)	20
Grey-headed	EN	Campbell Island	Campbell	NZ	6600	N	B, C	SIA (NB)	3
Grey-headed	EN	Îles Nuageuses	Kerguelen	France	7860	N	A	N	1
Grey-headed	EN	Isla Bartolome	Diego Ramírez	Chile	10880	Y	N	N	0
Grey-headed	EN	Isla Gonzalo	Diego Ramírez	Chile	4122	N	A	N	3
Grey-headed	EN	Main Island	South Georgia	UK	5177	N	N	N	0

Common name	IUCN Status	Breeding Site	Breeding population	Jurisdiction	Annual Breeding pairs	>10% pop	Morphological	Biochemical	Total Studies
Grey-headed	EN	Marion Island	Prince Edward	South Africa	8807	N	A	SIA, SIA (NB)	8
Grey-headed	EN	Paryadin Peninsula north	South Georgia	UK	6721	N	N	N	0
Grey-headed	EN	Paryadin Peninsula south	South Georgia	UK	22058	Y	N	N	0
Indian Yellow-nosed	EN	Falaise d'Entrecasteaux	Amsterdam	France	27000	Y	F,Cr	SIA, SIA (NB)	4
Indian Yellow-nosed	EN	Île des Pingouins	Crozet	France	5800	Y	C,Cr	N	1
Indian Yellow-nosed	EN	Prince Edward Island	Prince Edward	South Africa	5234	Y	C	SIA (NB)	2
Laysan	NT	Laysan Island	Hawaii	USA	134835	Y	A	N	3
Laysan	NT	Midway Atoll	Hawaii	USA	479526	Y	A	SIA, SIA (NB)	5
Light-mantled	NT	Campbell Island	Campbell	NZ	1600	Y	B	N	1
Light-mantled	NT	Île de la Possession	Crozet	France	1019	N	C,Cr	SIA), SIA (NB)	7
Light-mantled	NT	Île de l'Est	Crozet	France	900	N	N	N	0
Light-mantled	NT	Macquarie Island	Macquarie	Australia	2125	Y	A	N	2
Light-mantled	NT	(Bird Island)*	South Georgia	UK	5000	?	A	SIA, SIA (NB)	4
Light-mantled	NT	(Jeanne d'Arc Peninsula)*	Kerguelen	France	<4000	?	N	SIA, SIA (NB)	2
Light-mantled	NT	(Not specified)*	Auckland	NZ	5000	?	N	N	0
Northern royal	EN	The Big Sister	Chatham	NZ	1893	Y	N	N	0
Northern royal	EN	The Forty-fours	Chatham	NZ	2692	Y	N	N	0
Northern royal	EN	The Little (Middle) Sister	Chatham	NZ	1159	Y	A	SIA (NB)	5
Salvin's	VU	Depot Island	Bounty	NZ	16139	Y	N	N	0
Salvin's	VU	Funnel Island	Bounty	NZ	5159	Y	N	N	0
Salvin's	VU	Molly Cap	Bounty	NZ	3353	N	N	N	0
Salvin's	VU	Penguin Island	Bounty	NZ	2203	N	N	N	0
Salvin's	VU	Proclamation Island	Bounty	NZ	2851	N	N	SIA (NB)	1
Salvin's	VU	Ruatara Island	Bounty	NZ	5313	Y	N	N	0
Salvin's	VU	Spider Island	Bounty	NZ	3750	N	N	N	0
Salvin's	VU	Tunnel Island	Bounty	NZ	2333	N	N	N	0
Short-tailed	VU	Minami-kojima	Senkaku Retto	Japan	52	N	N	N	0
Short-tailed	VU	Torishima	Izu Shoto	Japan	538	Y	N	N	0

Common name	IUCN Status	Breeding Site	Breeding population	Jurisdiction	Annual Breeding pairs	>10% pop	Morphological	Biochemical	Total Studies
Shy	NT	Albatross Island	Tasmania	Australia	4859	Y	A	SIA (NB)	6
Shy	NT	The Mewstone	Tasmania	Australia	10000	Y	N	N	0
Sooty	EN	Gough Island	Gough	UK	3750	Y	N	SIA (NB)	1
Sooty	EN	Île de l'Est	Crozet	France	1300	N	N	N	0
Sooty	EN	Marion Island	Prince Edward	South Africa	1469	Y	A	SIA (NB)	4
Sooty	EN	Prince Edward Island	Prince Edward	South Africa	1210	N	N	N	0
Sooty	EN	Tristan da Cunha	Tristan da Cunha	UK	2500	Y	B	N	1
Southern royal	VU	Campbell Island	Campbell	NZ	7855	Y	A	N	7
Tristan	CR	Gough Island	Gough	UK	1650	Y	C	SIA, SIA (NB)	3
Wandering	VU	Bird Island	South Georgia	UK	859	N	A	SIA, SIA (NB)	24
Wandering	VU	Île aux Cochons	Crozets	France	1060	Y	N	N	0
Wandering	VU	Marion Island	Prince Edward	South Africa	2050	Y	A	SIA, SIA (NB)	6
Wandering	VU	Prince Edward Island	Prince Edward	South Africa	1800	Y	C	N	1
Wandering	VU	Rallier du Baty Peninsula	Kerguelen	France	750	N	N	N	0
Waved	CR	Isla Espanola	Galapagos	Ecuador	9607	Y	A	SIA	3
White-capped	NT	Auckland Island	Auckland	NZ	5592	N	N	N	0
White-capped	NT	Disappointment Island	Auckland	NZ	94727	Y	N	SIA (NB)	1

* These IBS may not have greater than 5% of the breeding population, however population counts are unavailable. The total island counts are >5% of the population

Chapter 3 - Optimised scat collection protocols for dietary DNA metabarcoding in vertebrates

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"It starts with an "s" and it ends with a "t"
It comes out of you and comes out of me
I know what you're thinking, you can call it that
But let's be scientific and call it scat"

Andy Bennett, Mary Keebler, Rodd Pemble, Doug Elliott, Billy Jonas

3.1 Abstract

DNA metabarcoding of food in animal scats provides a non-invasive dietary analysis method for vertebrates. A variety of molecular approaches can be used to recover dietary DNA from scats; however, many of these also recover non-food DNA. Blocking primers can be used to inhibit amplification of some non-target DNA, but this may not always be feasible, especially when multiple distinct non-target groups are present.We have developed scat collection protocols to optimise the detection of food DNA in vertebrate scat samples. Using shy albatross (*Thalassarche cauta*) as a case study, we investigated how DNA amplification success and the proportion of food DNA detected are influenced by both environmental and physiological parameters. We show that both the amount and type of non-target DNA varies with sample freshness, the collection substrate, fasting period and developmental stage of the consumer. Fresh scat samples yielded the highest proportion of food sequences. Collecting scats from dirt substrates reduced the proportion of food DNA and increased the proportion of contaminating DNA. Food DNA detection rates changed throughout the albatross breeding season and related to the time since feeding and the developmental stage of the animal. Fasting albatross produced scats dominated by parasite amplicons in universal PCR analysis, with little food DNA recovered. Samples from very young animals also produced reduced food DNA proportions. Based on our observations, we recommend the following procedures for field scat collections to ensure high quality samples for dietary DNA metabarcoding studies. Ideally, i) collect fresh scats; ii) from surfaces with minimal contamination (e.g. rock or ice); iii) collect scats from animals with minimum time since feeding and avoid fasting animals; iv) avoid young animals that aren't feeding directly (e.g. not weaned or fledged) or target larger/older individuals. The optimised field sampling protocols that we describe will improve the quality of dietary data from vertebrates by focusing on samples most likely to contain food DNA. They will also help minimise contamination issues from non-target DNA and provide standardised field methods in this rapidly expanding area of research.

3.2 Introduction

Scat samples provide an important source of DNA that can be utilised in a wide range of molecular ecology studies (e.g. Davison et al. 2002, Prugh et al. 2005). Food DNA present in scats provides a non-invasive and increasingly popular tool for studying vertebrate diet and can be applied to both predators and herbivores (e.g. Deagle et al. 2009, Zeale et al. 2011, Bowser et al. 2013, Kartzinel et al. 2015). Dietary DNA metabarcoding uses high-throughput sequencing of small, highly variable DNA regions that survive digestion to identify food species (Pompanon et al. 2012). This may involve identification of a particular food species using species specific markers (Jarman and Wilson 2004); food within a taxonomic group using group specific markers (Jarman et al. 2004, Murray et al. 2011, Zeale et al. 2011); identification of all food taxa using universal metazoan markers (O'Rorke et al. 2012a, Jarman et al. 2013); or a combination of these approaches (Deagle et al. 2009, Bowser et al. 2013). However, characterising the entire diet requires 'universal' markers that are capable of amplifying DNA from any food species (King et al. 2008, Jarman et al. 2013).

Universal metazoan polymerase chain reaction (PCR) primers amplify from all eukaryotic DNA, but will inevitably also amplify unwanted DNA from non-food items (Deagle et al. 2009, O'Rorke et al. 2012a). Non-target DNA within the scat may originate from the animal being sampled, its parasites, gut flora; or contamination from external organisms such as insects and vegetation. These sources of DNA can dominate the sequences amplified from a sample, making detection of DNA from food items less effective. Sample sizes must consequently be increased to address the underlying questions of a study, increasing processing costs. In some cases, non-target DNA amplification can be reduced by using a blocking primer to suppress amplification of specific DNA types, such as DNA of the defecating animal (O'Rorke et al. 2012b). However, development of blocking primers is challenging and food sequences may be inadvertently blocked with this approach. The use of blocking primers becomes more complex when there are multiple non-target DNA groups present. Improved sampling procedures are another approach for increasing the proportion of food DNA identified in a scat.

Selective scat sampling to improve DNA amplification success in genotyping studies has been investigated (Lucchini et al. 2002, Piggott 2004, Panasci et al. 2011, Vynne et al. 2012), but studies to optimise scat collections for DNA dietary analysis are rare (Oehm et al. 2011). Genotyping studies have investigated how the age of scats (Farrell et al. 2000, Lucchini et al. 2002, Piggott 2004, Panasci et al. 2011, Vynne et al. 2012), habitat type (Vynne et al. 2012) and season (Lucchini et al. 2002, Piggott 2004) affect DNA detection and genotyping accuracy. Fresh scats collected in dry and cool

conditions typically provided the highest amplification success and lowest genotyping error rate. However, the time since an animal defecated is seldom known and proxies for scat age are often required. For example, in maned wolf (*Chrysocyon brachyurus*) scats, higher moisture content and odour was found to be positively correlated with amplification success (Vynne et al. 2012). Similarly in brush-tailed rock-wallaby scats (*Petrogale penicillata*), colour, consistency and odour correlated well with DNA amplification success (Piggott 2004).

Only one dietary DNA study has examined how field conditions can influence the detection of food DNA. In carrion crow (*Corvus corone corone*) scats, exposure to sunlight and rain over a five-day period caused significantly lower amplification success of food DNA (Oehm et al. 2011). This was exacerbated by dirt, which may increase the degradation of extracellular DNA (Levy-Booth et al. 2007). This study used species-specific markers, which do not amplify non-food DNA. There are currently no studies that investigate whether targeted sample collections improve the detection of food DNA by universal metazoan markers.

We used shy albatross (*Thalassarche cauta)* as a model to develop optimised field protocols for dietary DNA metabarcoding of scats. Albatross are a good example as they follow predictable behavioural patterns, where they return to the colony after feeding and fast on the nest during incubation. This makes scat samples accessible and tests of fasting effects possible. Albatross are known to eat a diverse range of food items, including jellyfish, cephalopods, fish and carrion (Cherel and Klages 1998). Universal metazoan PCR primer sets which amplify from all potential prey groups are therefore needed to screen for all food items. Albatross colonies present far from ideal laboratory conditions. Colonies are typically exposed to extremes of weather, with little or no vegetation cover. Sample degradation by UV and rain, is likely to reduce PCR amplification success of exposed scats (Oehm et al. 2011). Contamination from non-food DNA, such as insects, parasites and fungi will also reduce the proportion of food DNA detected. Colonies are often remote and expensive to access, on trips that are generally short and/or infrequent, so effective scat collection is imperative.

The optimised field protocols that we developed increase the detection of food DNA by considering the effect of sample freshness; the substrate it was collected from; the bird's breeding and developmental stage; and fasting time. The effects that these factors have on the detection of food DNA are significant enough to be an important consideration when designing dietary DNA studies of vertebrates.

3.3 Methods

3.3.1 Case study species

Shy albatross lay one egg from early September to early October. The egg is incubated for 10 weeks (incubation stage) and the hatched chicks are brooded for 3-4 weeks (brood stage). During these two breeding stages, parents alternate nest attendance and foraging trips. After brooding, chicks are left unattended while both parents forage independently at sea to complete chick rearing (chick-rearing stage; Hedd and Gales 2005). During incubation, foraging trips may last from one to ten days, with an average of three days (Hedd et al. 2001), therefore an incubating bird could be fasting for this period or longer. Foraging trip durations during the brood stage are short at around one day and increase slightly during chick-rearing to two-three days (Brothers et al. 1998, Hedd and Gales 2005).

3.3.2 Field methodology

Shy albatross scat samples were collected at Albatross Island, Tasmania, Australia (40°23'S, 144°39'E). Scat samples were collected during the breeding period over two seasons: 2013/14 austral summer, chick-rearing (late March) only; and 2014/15 austral summer: incubation (late September), brood stage (mid December) and chick-rearing (late March). Samples were collected during the daytime from albatross observed defecating. A small fragment of the non-uric acid portion of the scat (dark part) was collected using tweezers or a plastic straw. The sample was stored in 80% ethanol and shaken on collection to mix with the ethanol. The only time fresh scats were not collected was when sample freshness was investigated (see below).

1. Sample freshness

To determine the effect of sample freshness on DNA amplification rates and the proportion of food DNA detected, scats were collected during the chick-rearing period in 2013/14 and 2014/15. The amount of time a scat had been present was unknown when a scat was found. Consequently, we wanted to provide a proxy measure for freshness to allow selection of higher quality dietary material. To test this, scat samples were categorised as: 1) *'Fresh'* when the bird was seen defecating, 2) *'Recent'* when the scat was still wet but the bird was not seen defecating (there was often a skin forming on these scats), or 3) '*Dry*' when scats were old and had no apparent moisture.

2. Substrate type

The dominant substrate from which the scat was collected was recorded for all fresh scats collected during chick-rearing. Substrate categories included: dirt, rock and vegetation.

3. Breeding stage

To determine if collecting at different stages of breeding affected the results, we randomly collected from birds in the colony that we saw defecating during incubation, brood guard and chick-rearing of the 2014/15 breeding season.

4. Developmental stage

When known, the breeding cohort of the bird was recorded as either 'breeder' a bird on an active nest or seen feeding a chick; 'non-breeder' a bird at an empty nest; or 'chick' which could have been a brooded chick <2 weeks old, or a pre-fledged chick ~3.5 months old.

5. Fasting

To test the effect that fasting had on dietary results, additional scats were collected during incubation. Two study sites within the colony were set up, each containing approximately 100 nests. Each bird was marked on the chest with a small dot of non-toxic stock-marker, with a different colour used to identify their partner to monitor the amount of time a bird had been incubating. Nests were numbered and checked daily at each site and the bird incubating recorded. When birds were observed defecating, the scat sample was collected and the nest number and bird colour was recorded. The incubation time was categorised as < 1 day, 1-2 days and >2 days.

3.3.3 DNA metabarcoding

Sample storage and extraction

Samples were stored at 5-10°C for one week whilst in the field, then -20°C until DNA was extracted. DNA was extracted within two weeks of collection using a Promega Maxwell 16 instrument and a Maxwell® 16 Tissue DNA Purification Kit (Madison, WI, USA). Samples were vortexed prior to extraction and ~ 30 mg of each sample was used. The quantity was consistent across extractions, which were all performed by the same person. PCR inhibitor concentrations were reduced in the DNA by mixing this sub-sample in 250uL of STAR buffer (Roche Diagnostics, Basel, Switzerland) prior to extraction.

PCR amplification and amplicon sequencing

A PCR primer set for amplifying ~170bp of the V7 region of the nuclear small subunit ribosomal DNA gene (18s; Hadziavdic et al. 2014) was designed manually on an alignment of the region that incorporated representatives from all major animal lineages. A two-stage PCR process was used to

enable amplification of the DNA region and attachment of unique 'tag' sequences to each sample which allows amplified samples to be pooled (Binladen et al. 2007).

Stage one PCR reactions (10 uL) were performed with 5 uL 2x Phusion HF (NEB, Ipswich, MA, USA), 1 uL 100x Bovine Serum Albumin (NEB), 0.1 uL 5 uM of each 18s_SSU amplification primer (Table 3.1), 0.5 ul of Evagreen, 2uL faecal DNA and 1.3 uL of water. Thermal cycling conditions were 98^{o} C, for 2 mins; followed by 35 cycles of 98^{o} C for 5s, 67^{o} C for 20 s, 72^{o}C for 20 s, with an extension of 72^{o} C for 1 min. Each sample was run in triplicate on a LightCycler 480 (Roche Diagnostics). A negative control containing no template DNA and positive control containing fish DNA were included in each PCR amplification run. If either the negative amplified or the positive failed to amplify, the PCR was re-run. Samples from each experiment were split among different PCR runs to avoid run-specific biases.

If ≥ 2 replicates of each sample had a 'crossing threshold' (ct) score < 30 they were combined to reduce biases produced by amplification from low template concentration samples (Murray et al. 2015). Pooled samples were diluted 1:10 for the second stage PCR. A unique tag was attached to each sample (Table 3.1) in 10 uL PCR reactions with 5 uL 2x Phusion HF (NEB), 1 uL 100 x Bovine Serum Albumin (NEB), 1 uL of 1 uM of each tag primer (Appendix 3.1), and 2 uL of diluted PCR product from stage one. Thermal cycling conditions were 98^{o} C, for 2 mins; followed by 10 cycles of 98^{o} C for 5 s, 55^{o} C for 20 s, 72^{o} C for 20 s, with an extension of 72^{o}C for 1 min. Four microlitres of PCR product from each sample (n=511) and the negative controls were pooled and purified from unincorporated reaction components by washing utilising reversible binding to Agencourt Ampure (Beckman Coulter, Brea, CA, USA) magnetic beads, with 1.8ul of Ampure per microlitre of DNA product. Sequencing of PCR products was performed with a MiSeq genome sequencer (Illumina, San Diego, CA, USA), using the MiSeq reagent kit V2 (300 cycles) with paired-end reads.

Table 3.1 Oligonucleotides used in this study. Underlined bases in PCR Round 1 are the Miseq tag primer. Bolded bases in PCR Round 2 are an example of the unique tags attached to each sample. A full list can be found in Appendix 3.1.

PCR Round	Primer Name	Primer sequence (5'-3')
1	18s_SSU_F	<u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u>GGTCTGTGATGCCCTTAGATG
1	18s_SSU_R	<u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u>GGTGTGTACAAAGGGCAGGG
2	SSU3_Tag_F1	AATGATACGGCGACCACCGAGATCTACAC**AGTTCGGACT**TCGTCGGCAGCGTC
2	SSU3_Tag_R1	CAAGCAGAAGACGGCATACGAGAT**AGCTTAGGCT**GTCTCGTGGGCTCGG

3.3.4 Bioinformatics

Amplicon pools were de-multiplexed based on unique 10 bp Multiplex IDentifiers (MIDs) on the MiSeq and fastq files processed using USEARCH v8.0.1623 (Edgar 2010). Reads R1 and R2 from the paired end sequencing were merged using the fastq_mergepairs function, retaining only merged reads flanked by exact matches to the 18S_SSU primers and primer sequences were trimmed. Reads from all samples were pooled and dereplicated, then clustered into broad Operational Taxonomic Units (OTUs) using the cluster_otus command (-otu_radius_pct = 10). Potentially chimeric reads are discarded during this step. Reads for each sample were assigned to these OTUs (usearch_global -id 0.9) and a summary table generated using a custom R script. Each OTU was assigned to a taxon by BLAST against a local database derived from the SILVA SSU database release 118 (Quast et al. 2013) with a 0.95 similarity used as a cutoff for identification. OTUs were categorised into seven groups based on their assumed origin: food, bird, parasite, fungi, plant, contaminant and unicellular (Appendix 3.2; Jarman et al. 2013). The contamination category included human, insect and ectoparasite sequences. Any sequences that did not match the Silva database were excluded from analysis (3.2% of the total). Although some species of albatross are known to eat birds, this has rarely been recorded in shy albatross (Hedd and Gales 2001), therefore in this study, the bird category represented DNA belonging to the albatross.

3.3.5 Statistical analysis

We assessed if DNA amplification success was affected by the specific variables (sample freshness, substrate, breeding stage, cohort and fasting length). Amplification was deemed successful if the total number of DNA sequences was >500 for a sample. We then examined whether there was a significant difference in the proportion of food DNA detected for each of the variables. Generalised linear models (GLMs) were used to test the difference in amplification success and quasi-binomial GLMs (to account for overdispersion in the data) were used to test differences in the proportion of food DNA detected (McCullagh and Nelder 1989). Analysis of deviance (with Chi-squared test) was used to test for significance of predictor terms, with post-hoc multiple comparisons by Tukey's method. Analyses were carried out using the R ‘stats’ package (R Core Team 2015), with multiple comparisons using the package 'multcomp' (Hothorn et al. 2008) and plots created using the package ‘ggplot2’ (Wickham 2009).

3.4 Results

DNA was extracted from 598 scat samples, with 511 of these producing ct values <30, with 458 (89%) producing >500 DNA sequence reads. A total of 2.9 million sequence reads were obtained from the single sequencing run, which included 452 305 (15.6%) food sequences (Figure 3.1).

3.4.1 Sample freshness

The freshness of scat samples significantly affected the DNA amplification success ($\chi^2_{2, 254} = 7.61$, $p = 0.02$), with fresh scats amplifying better than recent scats, but not better than dry scats (Table 3.2). Sample freshness also significantly affected the proportion of food DNA in the samples, ($\chi^2_{2,192} = 31808$, $p = 0.02$), with fresh scats containing a greater proportion of food DNA than dry scats, but not significantly more than recent scats (Table 3.2, Figure 3.2). Fungi DNA proportions were higher for dry scats than either fresh or recent (Figure 3.3).

3.4.2. Substrate type

Only a small number of scats were collected from vegetation, therefore substrate comparisons were only analysed using the two most common surfaces: dirt and rock. The substrate did not significantly affect amplification success ($\chi^2_{1,194} = 0.001$, $p = 0.97$), but did significantly affect the proportion of food DNA detected ($\chi^2_{1,148} = 14805$, $p = 0.04$). Scats obtained from rock contained a higher proportion of food DNA than those obtained from dirt (Table 3.2, Figure 3.2), which contained a higher proportion of unicellular DNA (Figure 3.3).

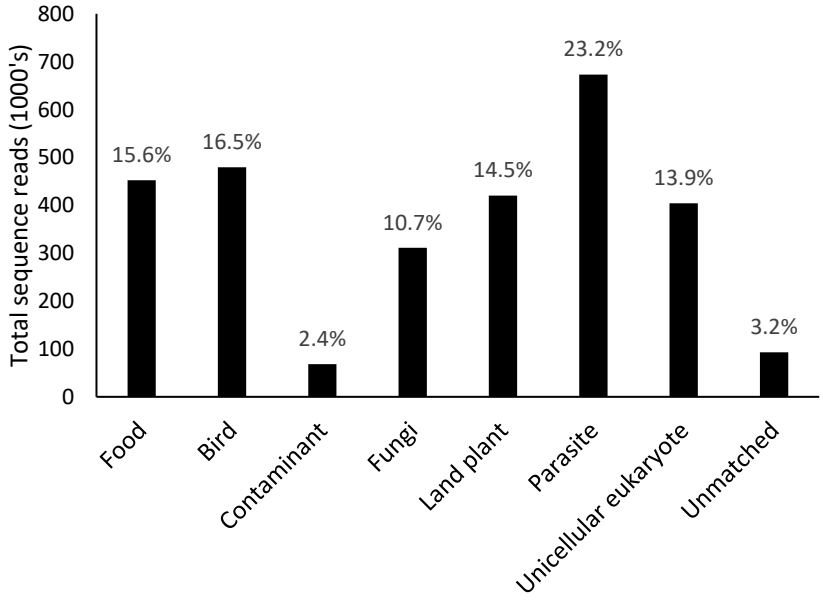

Figure 3.1 Total sequence reads obtained and categoried using the SILVA SSU database. Contaminants included insects (31 628 reads), ecto-parasites (31 578 reads) and human DNA from handling (5168 reads).

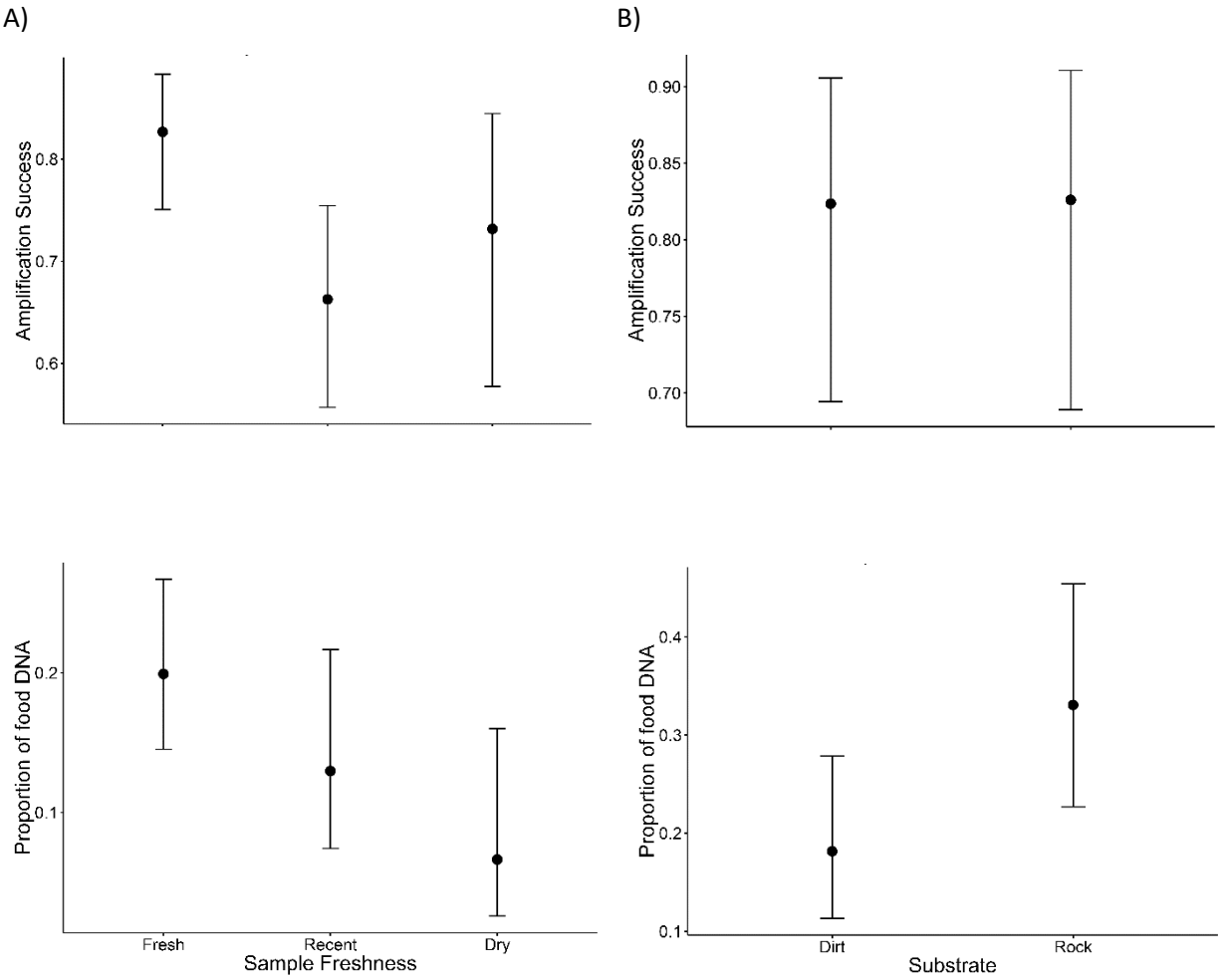

Figure 3.2 GLM fitted plots for: A) sample freshness (fresh, recent or dry) and B) substrate (dirt or rock). All scat samples collected during chick-rearing, with only fresh scats included in the analysis of substrate. Points represent means and bars show 95% confidence intervals.

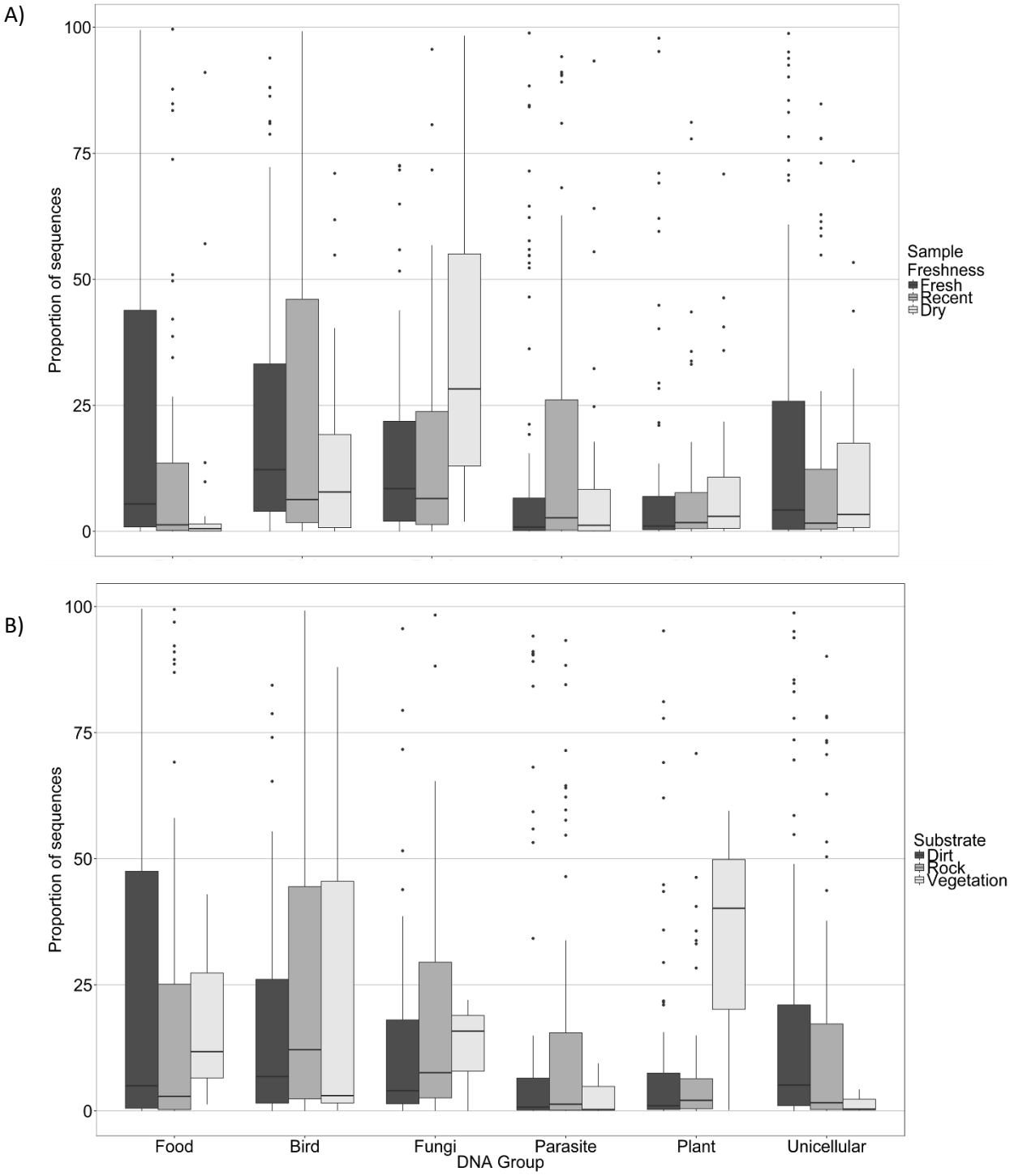

Figure 3.3 Sequence proportions of each DNA group for: A) sample freshness (fresh, recent and dry) and B) substrate (dirt, rock and vegetation). All samples collected during chick-rearing, with only fresh scats included in the analysis of substrate. To improve readability, the category 'contaminant' was excluded from the graph as it contained a very small proportion of DNA sequences.

3.4.3 Breeding stage

There was a significant difference observed in the DNA amplification success between breeding stages ($\chi^2_{2,308} = 7.988$, $p = 0.02$), with scats collected during the brood stage having lower amplification success (Table 3.2, Figure 3.4). The breeding stage greatly affected the proportion of food DNA detected ($\chi^2_{2,237} = 115863$, $p < 0.001$), with scats collected randomly during incubation producing significantly lower proportions of food DNA than scats from brood or chick-rearing stages (Table 3.2, Figure 3.4). Scats collected during incubation were dominated by parasites (98% cestoda; Figure 3.5).

3.4.4 Developmental Stage

During incubation, there was no significant difference between breeders and non-breeders in DNA amplification success ($\chi^2_{1,79} = 0.053$, $p = 0.82$; Table 3.2), or the proportion of food DNA detected in scats ($\chi^2_{1,69} = 11502$, $p = 0.09$; Table 3.2, Figure 3.4). However, during brood guard, the developmental stage of birds did significantly affect the DNA amplification success ($\chi^2_{2,166} = 8.711$, $p = 0.01$). Scats from chicks had a lower amplification success than those from breeders (Table 3.2, Figure 3.4). The proportion of food DNA detected was also significantly affected by the developmental stage during brood guard, ($\chi^2_{2,119} = 88972$, $p < 0.001$), with scats from breeders containing a much higher proportion of food DNA than those from chicks or non-breeders (Table 3.2, Figure 3.4). During the brood stage, chick scats had a higher proportion of bird, fungi and plant DNA than breeders, whereas non-breeder scats were dominated by parasites (Figure 3.5).

3.4.5 Fasting

The time a bird spent fasting did not significantly affect the DNA amplification success of the scat ($\chi^2_{3,178} = 3.01$, $p = 0.39$), but did strongly affect the proportion of food DNA detected within the scat ($\chi^2_{3,147} = 70165$, $p < 0.001$). Scats collected from birds incubating for less than a day had a far greater proportion of food DNA detected than scats collected randomly, however this was not the case for any other incubation length category (Table 3.2, Figure 3.5). Scats from birds that had been incubating longer than one day contained predominantly parasite DNA (Figure 3.5).

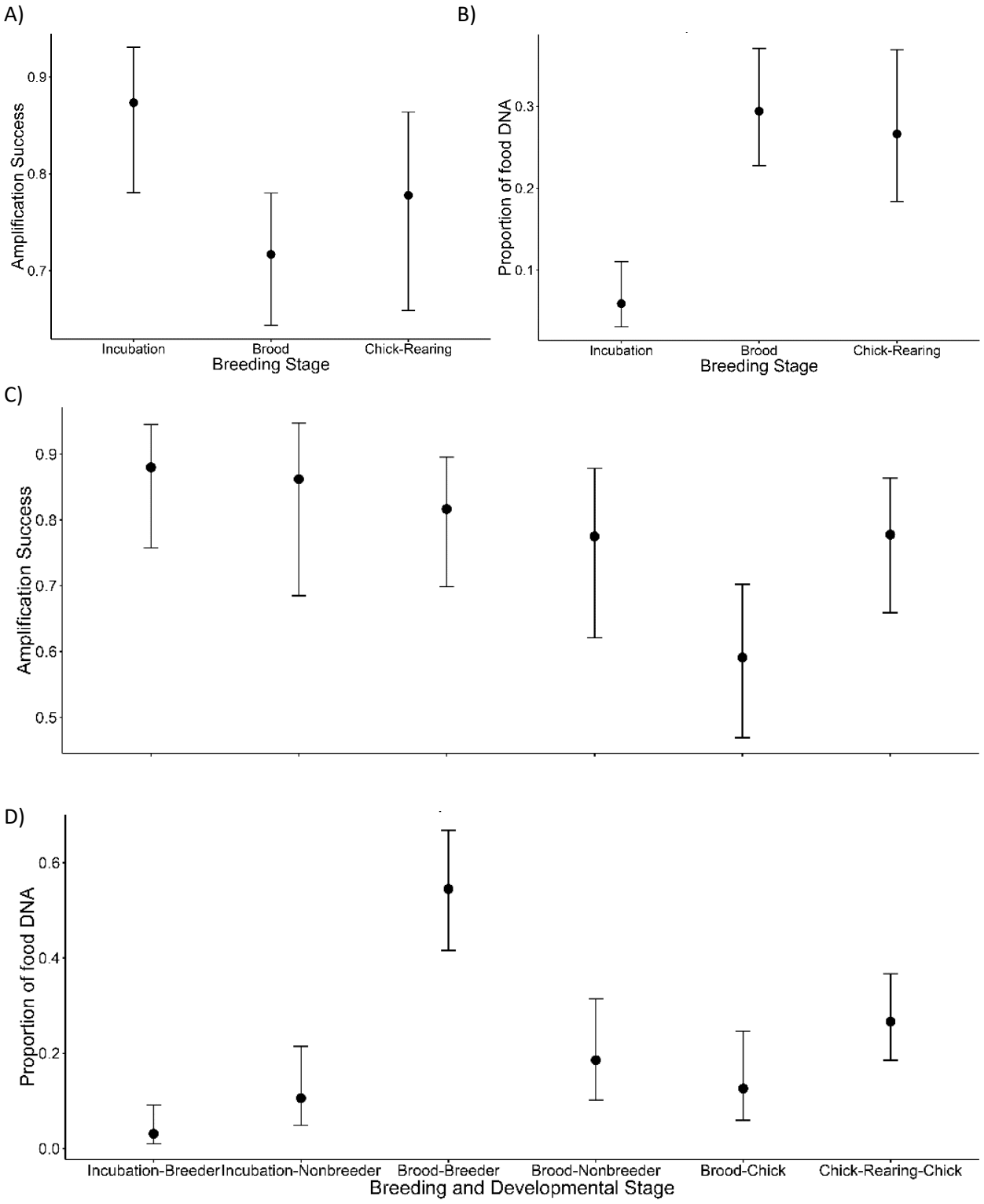

Figure 3.4 GLM fitted plots of the A) amplification success and B) proportion of food for each breeding stage (incubation, brood and chick-rearing); and C) the amplification success and D) the proportion of food for each age cohort within each breeding stage. Incubation samples included only scats collected randomly where incubation length was unknown. Points represent means and bars show 95% confidence intervals.

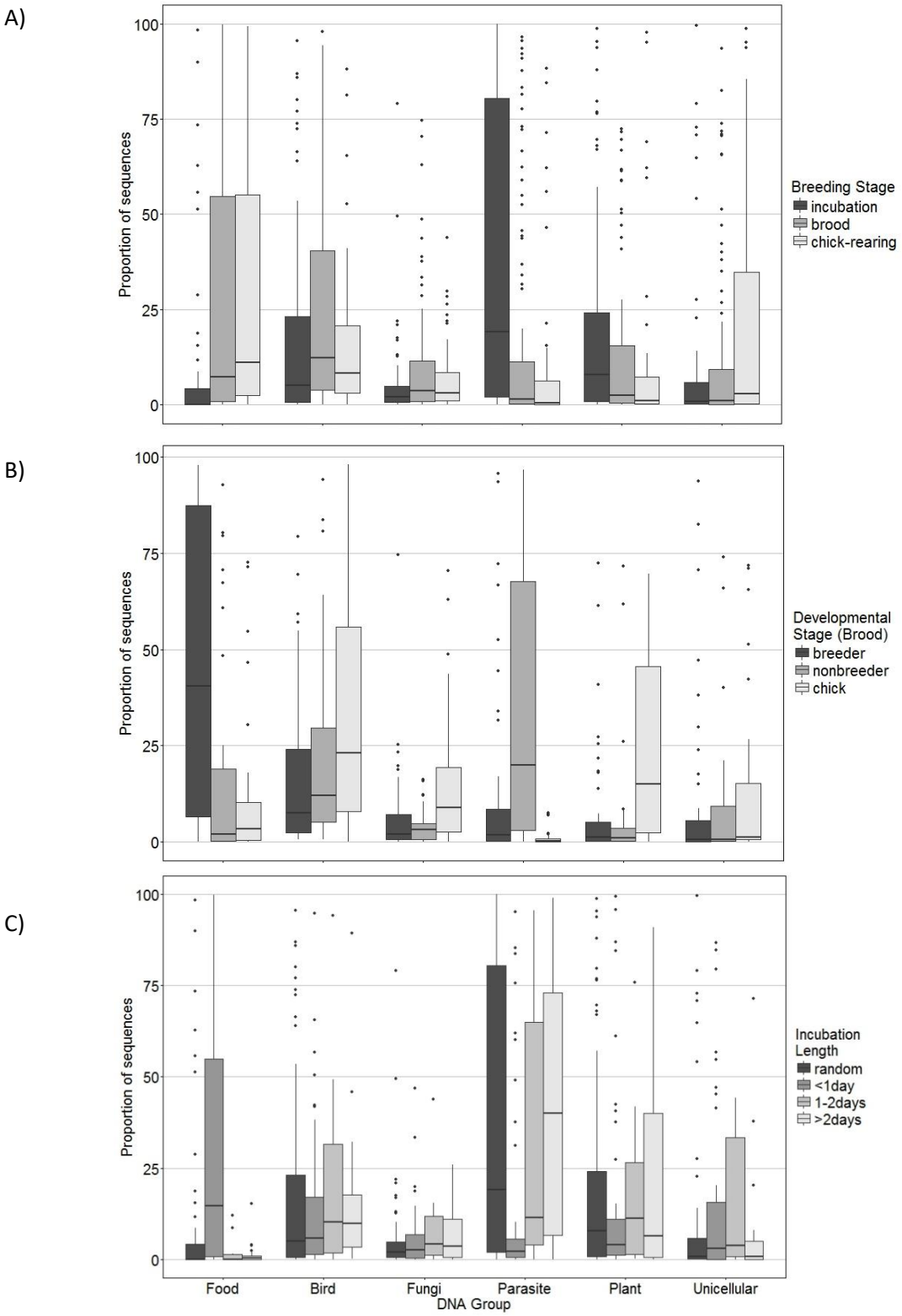

Figure 3.5 Sequence proportions for each DNA group for breeding stage, developmental stage and incubation length. Figure A) breeding stage (incubation, brood and chick-rearing); B) developmental stage during brood (breeder, chick and non-breeder); and C) Incubation length (random, <1 day, 1-2days and >2 days). Only fresh scats were analysed. To improve readability, the category 'contaminant' was excluded from the graph as it contained a very small proportion of DNA reads. The incubation category in 'A' included scats collected randomly where incubation length was unknown.

Table 3.2 Generalised linear model (GLM) outputs for comparisons of DNA amplification success and the proportion of food DNA detected for sample age, substrate, breeding stage, developmental stage and fasting time. DNA amplification was analysed using binomial GLMs, the proportion of food DNA using quasi-binomial GLMs. Superscript symbols indicate significantly different values (Tukey multiple comparison test) *p<0.05, $^{\#+}$ p<0.001.

				DNA Amplification			Proportion of food DNA		
		Scats obtained	Scats with DNA amplified	Amplification success	Estimated values	SE	Estimated values	St Error	Fitted
Sample freshness	Fresh	127	105	82.7	1.563*	0.234	-1.391*	0.193	0.20
	Recent	86	57	66.2	-0.887*	0.327	-0.513	0.369	0.13
	Dry	41	30	73.2	-0.560	0.423	-1.253*	0.536	0.07
Substrate	Dirt	90	70	77.8	1.540	0.367	-1.505*	0.278	0.18
	Rock	104	78	75.0	0.017	0.535	0.800*	0.382	0.33
Breeding Stage	Incubation	79	69	87.3	1.931*	0.338	$-2.768^{+\#}$	0.346	0.59
	Brood	166	119	71.7	-1.002*	0.380	1.893^{+}	0.388	0.29
	Chick-Rearing	63	49	77.8	-0.678	0.454	$1.755^{\#}$	0.423	0.27
Incubation cohort	Breeder	50	44	88.0	1.992	0.435	-3.439	0.649	0.03
	Non-Breeder	29	25	86.2	-0.159	0.692	1.306	0.803	0.11
Brood cohort	Breeder	60	49	81.7	1.494*	0.333	$0.180^{+\#}$	0.230	0.54
	Non-Breeder	40	31	77.5	-0.257	0.505	-1.659^{+}	0.386	0.19
	Chick	66	39	59.1	-1.126*	0.417	$-2.117^{\#}$	0.429	0.13
Incubation Time	Random	79	69	87.3	1.932	0.338	-2.769^{+}	0.318	0.06
	<1day	52	41	78.8	-0.616	0.479	1.639^{+}	0.415	0.24
	1-2days	18	13	72.2	-0.976	0.626	-1.928	2.108	0.01
	>2days	29	24	82.7	-0.363	0.597	-1.087	1.174	0.02

3.5 Discussion

Our case study clearly indicates that sample freshness, the substrate the scat was collected from, breeding stage, developmental stage and fasting can all impact the amount of food DNA available for dietary DNA metabarcoding. The scat collection protocol presented here contributes to optimising the amount of food DNA that is identified in vertebrate dietary studies.

3.5.1 Sample freshness

Scat freshness was found to affect both the DNA amplification success and the proportion of food DNA detected. Fresh scats exhibited a higher DNA amplification success than recent scats, but not dry scats. We had expected that both recent and dry scats would have less amplifiable DNA than fresh scats due to degradation during environmental exposure (Oehm et al. 2011). However, dry scats have also had more potential exposure to external contamination, particularly from fungi, which was reflected in the non-food DNA sequences recovered. When we look specifically at the amount of food DNA amplified from dry scats, this component was significantly less than for fresh scats. Although recent scats had a lower amplification success, the proportion of food DNA detected was not significantly lower than that of fresh scats. This 'recent' category contained a wide range of scats, from those defecated within minutes (but not seen), to those exposed for many hours. Therefore using scats that are still wet may produce dietary information, but larger sample sizes would be required and reliance on small amounts of DNA may reduce data quality (Murray et al. 2015).

Samples in this study were collected during the day from a species breeding in an exposed habitat with little protection from UV and rain. Scats collected in protected conditions such as from a shaded area, at night, or collected in the early morning may allow amplifiable DNA to persist for longer. For example, in carrion crows, food DNA could be detected for up to five days when protected from UV and rain exposure (68% success), however, this was dramatically reduced when scats were left in exposed areas (17.5% success; Oehm et al. 2011). Similarly, Steller sea lion (*Eumetopias jubatus*) scats also produced detectable prey DNA for up to five days in some samples (Deagle et al. 2005). However, in both of these studies, group specific markers were used that detected only food items. In our study, some dry scats still contained food DNA, so it is possible that with the use of group specific markers, dietary information may be detectable for longer.

To ensure fresh scats are collected in the field, some studies have captured or contained animals (Kartzinel and Pringle 2015, Lopes et al. 2015), or placed sheets to collect fresh faeces (Deagle et al. 2010, Vesterinen et al. 2016). When manipulation of the surrounding environment is not physically or ethically possible, alternative sampling strategies are required. In optimising scat collections, we did not seek to determine the amount of time in hours or days that a scat could be collected, as this information is unknown when a scat is found. Instead, we wanted to provide a proxy that allows field biologists to selectively collect scats that will provide high quality dietary material. Unfortunately, wet recent scats did not provide as much data as fresh scats, which meant that observing defecating animals will still be best practice in exposed locations. However, this is often not possible and other proxies may be required to determine sample freshness (e.g. odour, colour, consistency), as well as understanding how these may change between species, seasons and environments (Piggott 2004, Vynne et al. 2012, Demay et al. 2013).

Given the proportions of broad categories of DNA change as the sample ages it is possible that the measured proportions from various diet species in the samples may change too if DNA from different species degrades at different rates. This should be examined with experimental studies and care taken to ensure consistent collection methods between sites.

3.5.2 Substrate type

Scats collected from rock and dirt had similar amplification success, but scats from rock had a higher proportion of food DNA detected. This is partially consistent with Oehm et al (2011) who also found that carrion crow scat samples collected from dirt had reduced food DNA detectability, in both protected and exposed samples. However, they found that DNA detection was hampered by an increase in PCR inhibitors. This did not appear to affect the samples in our case study as amplification success was similar between dirt and rock samples. Instead, the presence of non-food DNA was higher in scats obtained from dirt. Our scats were fresh, compared to five day old scats from carrion crows; therefore the DNA in our samples may not have been as degraded. Shy albatross scat samples from dirt contained a higher proportion of unicellular DNA than from rock. Unicellular eukaryotes are common in soil and these sequences are likely to represent contamination. It is often difficult to separate scat samples from dirt, especially for very liquid samples that have been mixed into the dirt. Seabird colonies can be home to greater densities of microbial communities within the soil (Wright et al. 2010), which may exacerbate the presence of non-food DNA.

The three scats collected from plants were dominated by plant DNA. Any surface that contains DNA is likely to decrease the amount of food DNA due to increased contamination. An additional complication occurs when the substrate could be incorrectly assigned as a food item. This is particularly relevant for dietary studies on herbivore species when scats are collected from vegetation (Kartzinel et al. 2015), or marine species when scats are collected from the water (Jarman and Wilson 2004). If collecting from vegetation, the substrate species should be recorded to allow appropriate categorisation when interpreting sequencing results.

3.5.3 Breeding and developmental stage

Digestion rates are likely to vary for numerous reasons e.g. predator species, metabolic rate, meal size, food type and feeding frequency (Hilton et al. 1998). These may all in turn impact the detectability of food DNA in scats. Understanding how feeding behaviour may change throughout the year or breeding season for different developmental stages will impact how and when samples can be collected.

Collections from young animals are likely to pose problems for DNA dietary analysis depending on the way they obtain food. In this case study, young chicks had a lower proportion of food DNA detected than breeding adults and a higher proportion of bird DNA. In many avian species, juvenile food is delivered by regurgitation, therefore food items are likely to be partially digested before they are fed to the chick. This was the case in white-chinned petrels (*Procellaria aequinoctialis*), where food in chicks' stomachs were more digested than that of adults (Connan et al. 2007). Consequently, digestive processes may excessively degrade food DNA in chick scat samples. Additionally, there is presumably cross-over of parental DNA to the chick during regurgitation, which may cause the amount of bird DNA to be inflated, thereby reducing the food DNA proportionately. Interestingly, the converse results were seen with *Adélie* penguins (*Pygoscelis adeliae*), with scats collected from chicks more successful than those from breeders, especially during brood guard when chicks were small. Although a similar marker region was used in both studies, a blocking primer was used to suppress bird DNA amplification, which may explain this result (McInnes et al. 2016a).

Scat samples from young vertebrates should ideally be collected when they are directly feeding on the food themselves, rather than through secondary means. For birds fed by regurgitation, this may not be possible during the nestling period, however samples from older chicks did contain more food DNA. Older shy albatross chicks had a higher food proportion than small chicks, which may reflect larger meals or a reduction in stomach oil. Procellariiforme (albatross and petrel) stomachs contain

oil that is obtained from digested prey (Imber 1976). This oily liquid can contribute up to 80% of the sample mass in some albatross stomachs (Thompson 1992). In shy albatross, there is a greater mass of oily liquid in younger chicks than older chicks (Hedd and Gales 2001), which may dilute the food DNA. Young animals with diet supplemented by suckling milk could also have the same issue.

We also observed differences in food detection between breeding cohorts, with lower proportions of food DNA and higher proportions of parasite DNA detectable from scats of non-breeding animals during brood-guard. A non-breeder was identified by its presence at an empty nest and is likely to be either a failed breeder or sub-adult bird defending a nest. As these individuals do not need to forage to feed a chick, they may have been ashore longer and therefore could fall into a similar category to fasting/incubating birds (see below). This finding highlights the need to understand not only the biology of the study species, but also awareness of which breeding cohorts may be present during scat collections and how this may affect results.

3.5.4 Fasting

The detection of food DNA throughout the season was strongly linked to fasting. Longer periods of fasting during incubation resulted in a low detection of food DNA in scats, whereas food DNA detection was much higher for breeding birds during brood. This is likely to be linked to more frequent feeding trips during this stage. During periods of fasting, non-target DNA was dominated by endoparasites, rather than external contamination. Cestodes are the main endoparasites in pelagic seabirds and their presence is largely driven by diet and the availability of intermediate hosts e.g. zooplanktonic organisms and fish (Hoberg 1996). Interestingly, there was an apparent increase in parasite DNA during fasting. If the food DNA proportion alone had decreased, then it would be expected that all other DNA groups would increase proportionally. However, there appeared to be a greater increase in the parasite DNA than other groups, suggesting there was an increase in prevalence, not just detection. The exact cause of the increase during fasting is unknown, however care should be taken when obtaining scats, targeting animals with minimal time since feeding.

Fasting periods occur in many species for many reasons, including territory defence, hibernation, meal availability, migration, incubating or suckling young, moult or limited mobility e.g. during pregnancy. Understanding when these fasting periods occur in the study species is important for detection of dietary DNA in scats. Although defecation rate does slow during fasting, it often won't cease. Therefore, the risk of collecting scats that contain no dietary information needs to be taken into consideration when planning a study.

3.5.5 Field protocols for DNA scat collection

We have developed a method to allow high quality dietary information to be obtained using universal metazoan markers by optimising collection protocols, enabling a reduction in signal from non-target DNA.

Careful planning of DNA dietary metabarcoding studies prior to sample collection is imperative for overall project success. Researchers should consider the dietary question they are targeting and focus on which scat samples will inform this. This includes marker selection, seasonal changes, fasting and the age of animals. These considerations, especially animal behaviour and developmental stage, are likely to be important to a broad array of molecular ecology studies reliant on DNA in scat samples, or those using eDNA. To improve the quality and quantity of dietary information obtained from scat samples the following collection protocols should be followed when possible.

- Collect fresh scats where the animal is seen defecating. If this isn't possible, try to collect only scats that still have moisture or develop species specific proxies that correlate to sample age.
- Give serious consideration to the scat substrate type, as contamination from substrate can overwhelm the food DNA signal. Ideally, collect scats from surfaces with minimal sources of DNA contamination (e.g. rocks or ice). If collecting from dirt or vegetation, try to minimise the collection of foreign material and record the substrate (and species where applicable) to cross-check and validate results.
- Take into consideration the seasonal behaviour and feeding ecology of the study animal prior to sample collection.
- Avoid collections from animals that may not have fed recently, such as periods of fasting.
- Collect from animals that are directly feeding themselves and avoid secondary feeding where possible (including suckling young). Samples from young animals that are being fed by regurgitation may be problematic due to partially digested food passed on by the parents or large amounts of parental DNA. For such species, collection from older animals may be preferable.
- If only a single collection is available and the seasonal timing and cohort aren't the focus of the study, target the time period with the shortest time since feeding and focus on adult animals.
- If multiple study sites are used, keep collection protocols and timing consistent between sites

These optimised scat collection protocols provide a basis for future experimental designs and will enable ecologists to collect high quality diet samples and reduce non-target DNA amplification. They

also provide standardised field methods which will be important in this rapidly expanding area of research.

3.6 Acknowledgements

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3.7 Data accessibility

Data is accessible from the Australian Antarctic Data Centre (doi:10.4225/15/57EDACF067ADF).

3.8 Appendices

Appendix 3.1: Second round PCR unique tag combinations.

Reverse tag combinations		Forward tag combinations	
R1	AGCCTAAGCT	F1	AGCCTAAGCT
R2	GACCTGGACT	F2	AGTTCAAGTC
R3	CAGGTCCAGT	F3	ACTTGAACTG
R4	ACGGTAACGT	F4	ACGGTAACGT
R5	CGAATCCGAT	F5	ATCCGAATCG
R6	GCAATGGCAT	F6	ATGGCAATGC
R7	ACTTGAACTG	F7	CAGGTCCAGT
R8	CATTGCCATG	F8	CATTGCCATG
R9	CTAAGCCTAG	F9	CTAAGCCTAG
R10	ATCCGAATCG	F10	CGAATCCGAT
R11	GCTTAGGCTA	F11	TCAAGTTAGC
R12	CGTTACCGTA	F12	TACCGTTACG
R13	GTCCAGGTCA	F13	TGAACTTGAC
R14	CTGGACCTGA	F14	TAGGCTTCAG
R15	CTAAGAAGCT	F15	AGCCTCCAGT
R16	ATGGCAAGCT	F17	AGTTCGGACT
R17	AGTTCGGACT	F18	AGTTCTTGAC
R18	CTGGAGGACT	F19	ACGGTCCATG
R19	CATTGAACGT	F20	ACTTGTTCAG
R20	GTCCACCAGT	F21	ACTTGCCGAT
R21	ATCCGCCAGT	F22	ACGGTGGATC
R22	GATTCAACGT	F23	ATCCGCCTAG
R23	ACTTGCCGAT	F24	ATCCGTTAGC
R24	GCTTACCGAT	F25	ATGGCGGTAC
R25	GTAACGGCAT	F25	ATGGCGGTAC
R26	CGTTAGGCAT	F26	ATGGCTTACG
R27	GACCTAACTG	F27	CAGGTAAGTC
R28	ATGGCAACTG	F28	CAGGTGGCAT
R29	AGCCTCCATG	F30	CTAAGTTCAG
R30	CTGGACCTAG	F31	CTAAGAACGT
R31	ACGGTCCTAG	F32	CTGGATTGAC
R32	GTCCATTCAG	F33	CATTGTTAGC
		F34	CTGGAGGACT
		F35	CGAATAACTG
		F36	CGAATGGATC

Appendix 3.2- Categories of sequences assigned to each group. This contains the taxonomic order or class that was assigned to each of the seven broad categories: food, bird, contaminant, fungi, parasite, plant and unicellular.

Food	**Contaminant**	**Fungi**
Actinopterygii	Ectoparasites	Blastocladiomycota
Cephalopoda	Arcariformes	Chytridiomycota
Chondrichthyes	Parasitiformes	Entomophthoromycotina
Decapoda	Insecta	Ascomycota
Copepoda	Collembola	Mucoromycotina
Ascidiacea	Annelida	Basidiomycota
Haptophyta	Tardigrada	
Scyphozoa	Filasterea	**Parasite**
Hydrozoa	Primates (human)	Cestoda
Appendicularia		Nematoda
Euphausiacea		Trematoda
Rotifera	**Unicellular eukaryotes**	Myxozoa
Porifera	Alveolata	Heterophryidae
Maxillopoda	Stramenopiles	Mesomycetozoea
Rhodophyceae	Rhizaria	Rhombozoa
Bivalvia	Amoebozoa	Monogenea
Ctenophora	Excavata	Turbellaria
Cirripedia	Choanoflagellates	
Isopoda	Glaucophytes	**Plants**
Gastropoda	Cryptophyceae	Embryophyta
Anthozoa	Chlorophyceae	
Myriapoda	Trebouxiophyceae	**Bird**
Branchiopoda	Ulvophyceae	Archosauria
Nemertea	Prasinophytae	
Echinodermata	Mamiellophyceae	
Ostracoda	Chlorophyta spp	

Appendix 3.3: Field collection protocols for DNA dietary analysis of seabird scats.

This video provides an overview of the equipment, methods and guidelines for scat collections based on results presented in this chapter

https://youtu.be/CYFj2YZzKf0

A second video describes more extended protocols for seabird scat collections

https://youtu.be/CQ_6bUX91ls

Chapter 4 - High occurrence of jellyfish predation by black-browed and Campbell albatross identified by DNA metabarcoding

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"What we see before us is just one tiny part of the world. We get in the habit of thinking, this is the world, but that's not true at all. The real world is a much darker and deeper place than this, and much of it is occupied by jellyfish and things."

Haruki Murakami
The Wind-Up Bird Chronicle

4.1 Abstract

Gelatinous zooplankton are a large component of the animal biomass in all marine environments, but are considered to be uncommon in the diet of most marine top predators. However, the diets of key predator groups like seabirds have conventionally been assessed from stomach content analyses, which cannot detect most gelatinous prey. As marine top predators are used to identify changes in the overall species composition of marine ecosystems, such biases in dietary assessment may impact our detection of important ecosystem regime shifts. We investigated albatross diet using DNA metabarcoding of scats to assess the prevalence of gelatinous zooplankton consumption by two albatross species, one of which is used as an indicator species for ecosystem monitoring. Black-browed and Campbell albatross scats were collected from eight breeding colonies covering the circumpolar range of these birds over two consecutive breeding seasons. Fish was the main dietary item at most sites, however cnidarian DNA, primarily from scyphozoan jellyfish was present in 42% of samples overall and up to 80% of samples at some sites. Jellyfish was detected during all breeding stages and consumed by adults and chicks. Trawl fishery catches of jellyfish near the Falkland Islands indicate a similar frequency of jellyfish occurrence in albatross diets in years of high and low jellyfish availability, suggesting jellyfish consumption may be selective rather than opportunistic. Warmer oceans and overfishing of finfish are predicted to favour jellyfish population increases and we demonstrate here that dietary DNA metabarcoding enables measurements of the contribution of gelatinous zooplankton to the diet of marine predators.

4.2 Introduction

Gelatinous zooplankton (including scyphozoans, salps, ctenophores and hydrozoans) form a large biomass component of marine ecosystems and are thought to be increasing in abundance in some areas (Brodeur et al. 2002, Richardson et al. 2009, Brotz et al. 2012). Jellyfish have traditionally been regarded as an unlikely primary prey source because of their very low energy density, especially in comparison to common alternative prey groups like fish (Doyle et al. 2007). There is growing evidence that these gelatinous animals are consumed by many larger animals either through predation or scavenging (Houghton et al. 2006, Cardona et al. 2012, Milisenda et al. 2014, Sweetman et al. 2014). However, consumption by seabirds has only been observed intermittently, involving direct observations of predation (Fraser 1939, Weimerskirch et al. 1986, McCanch and McCanch 1996, Arai 2005, Suazo 2008) or analysis of stomach contents of birds caught or killed at sea, rather than at breeding colonies (Harrison 1984, Schneider et al. 1986, Arai 2005). Gelatinous organisms are difficult to identify in stomach contents samples using visual identification of prey remains because they lack robust diagnostic morphological features and are rapidly digested (Arai et al. 2003). Consequently, hard parts of animals such as cephalopod beaks, fish bones and crustacean carapaces are more likely to be represented in stomach content samples (Barrett et al. 2007). This issue is compounded by the retention of some prey parts in the stomach, for example squid beaks can be retained for up to 50 days in albatrosses (Furness et al. 1984).

In recent years, the ability to detect gelatinous prey consumption by seabirds has improved through the use of animal-borne cameras (Sutton et al. 2015, Thiebot et al. 2016) and DNA metabarcoding of scat samples (Jarman et al. 2013, McInnes et al. 2016a). DNA dietary metabarcoding can identify prey DNA in predator scats without biases from retention of hard-parts and can detect soft-bodied prey (O'Rorke et al. 2012a, Pompanon et al. 2012). Using these methods, scyphozoan jellyfish have been detected frequently in the diet of Adélie penguins (Jarman et al. 2013, McInnes et al. 2016a). However, the role of jellyfish as a prey item remains unclear for many seabird predators. It is not known, for example, whether jellyfish are taken opportunistically or as a targeted prey; or if they are more important as a prey item during certain times of the year. If consumption of gelatinous prey is opportunistic, it might be expected that their prevalence in the diet would follow prevalence in the foraging region. Higher jellyfish abundances would lead to more frequent encounters and therefore higher occurrence in the diet. To subsist largely on jellyfish requires predators to consume large volumes (Duron 1978), which may be possible when jellyfish occur in large groups or hotspots (Houghton et al. 2006).

The duration of seabird foraging trips is constrained during the breeding season by the need to return to the nest to provision chicks. These constraints can be met using a variety of foraging strategies, including parents minimising energy expenditure by selecting higher quality prey for provisioning compared to self-feeding (Ydenberg et al. 1994). Thus, it is possible that gelatinous prey might typically be consumed during adult self-feeding, rather than for provisioning chicks. Since the majority of seabird diet studies are conducted during chick rearing and represent the provisioning diet (Barrett et al. 2007, McInnes et al. 2016b - Chapter 2), the prevalence of gelatinous prey would naturally be low in these studies.

Understanding the full spectrum of seabird diets is important not only to investigate the foraging ecology of the bird, but also to assess the potential impacts of threats such as climate change and fishing, and thus has implications for the way we undertake ecosystem monitoring. The hierarchical nature of food-webs means that the diets of top order predators such as seabirds are responsive and reflective of overall change in availability of lower trophic levels (Boyd and Murray 2001). Marine ecosystems are difficult to study due to their relative inaccessibility and therefore top predator diet is often used to identify changes in the overall species composition of an ecosystem, including the availability of different prey groups (Croxall et al. 1999, Chiaradia et al. 2010). However, if the dietary methods used to assess these changes cannot accurately identify all trophic connections then the interpretation of dietary results could be misleading.

Albatrosses are one of the most threatened seabird groups because they are incidentally killed (bycaught) by commercial fisheries and affected by environmental change (Phillips et al. 2016). The black-browed albatross (*Thalassarche melanophris*) is one of the most numerous albatross species and breeds on 14 island groups, with a circumpolar distribution (ACAP 2010). Black-browed albatross diet has been well studied compared to that of other albatross species (McInnes et al. 2016b - Chapter 2), and they are used as an indicator species in ecosystem monitoring (SC-CCAMLR 1997). The Campbell albatross (*Thalassarche impavida*) is closely related and is endemic to Campbell Island, New Zealand. There have been 12 papers reporting the complete diet from black-browed albatross stomach contents, which equates to 18 studies when stratified by year and site. The main prey groups identified from stomach contents are fish and cephalopods and gelatinous prey have only been recorded in 8% of published papers (n=1) and 16% of studies (n=3), all from the Falkland Islands. In these studies, jellyfish were detected infrequently (<20% of samples) and in low volumes (< 5.3% by mass; Thompson 1992). The single study on Campbell albatross diet also reported gelatinous organisms as a minor prey item (< 2.3% by prey mass; Cherel et al. 1999). Despite the rare

occurrence in stomach contents predation of scyphozoan jellyfish has been observed visually in black-browed albatross (Weimerskirch et al. 1986, Suazo 2008), and stomach temperature loggers and stable isotopes used on species in the same genus indicate their consumption may be more common (Catry et al. 2004, Connan et al. 2014).

In this study, we examined the prevalence of gelatinous prey in the diet of black-browed and Campbell albatross. We also estimated the relative availability of jellyfish from net catches by fishery vessels near two of the sites where we sampled albatross scats. We hypothesise that gelatinous prey commonly occur in the diets of albatross, but that consumption is likely to be opportunistic and reflect prey availability. We used DNA metabarcoding of albatross scat samples collected from eight colonies across their breeding range and spanning two breeding seasons to assess the prey groups consumed. We also assessed dietary differences between years, breeding sites and breeding stages.

4.3 Methods

4.3.1 Study sites and sample collection

A total of 1460 fresh black-browed albatross scat samples were collected from seven breeding colonies and Campbell albatross from one colony, over multiple seasons: in 2013/14 and 2014/15 at New Island and Steeple Jason Island (Falkland Islands), Macquarie Island (Australia), Campbell Island (New Zealand), and Bird Island (South Georgia); in 2013/14 and 2015/16 at Canyon des Sourcils Noirs (Kerguelen Island); in 2014/15 and 2015/16 at Albatross Islet (Chile); and in 2013/14 at Diego Ramírez (Chile; Figure 4.1). Most samples (n=1185) were collected during the chick-rearing period with 718 during early chick-rearing (early December to end of January) and 467 during late chick-rearing (February and March), an additional 275 samples were collected during incubation (October to early December, Appendix 4.1). Samples from chicks and adults were identified where possible. Due to the availability of birds at the colony, samples were predominantly collected from adults during incubation and early chick-rearing and chicks during late chick rearing. As such, samples sizes were too low during this study to directly compare dietary differences between chicks and adults; however, dietary comparisons between breeding stages were examined for sites where samples were collected during multiple breeding stages.

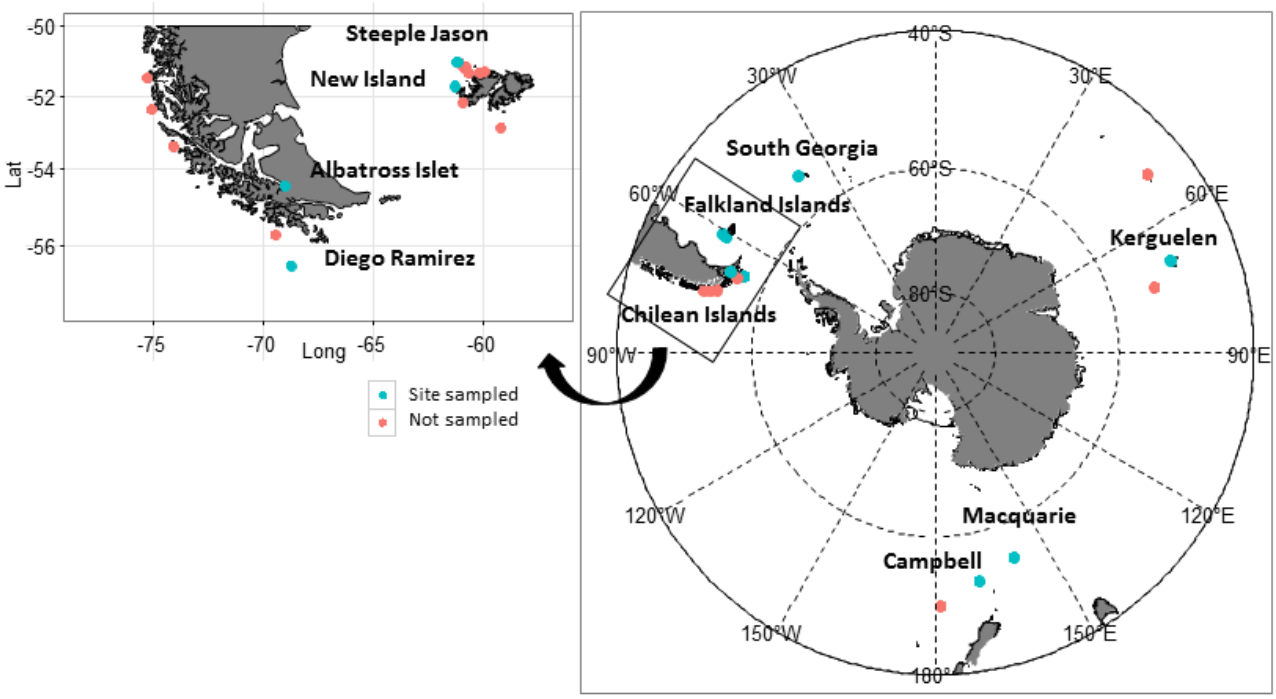

Figure 4.1 Breeding distribution of Black-browed and Campbell albatrosses. Blue dots represent the eight colonies where scat samples were collected, and the red dots the remaining colonies not sampled during the study. The inset shows the individual Chilean and Falkland Island colonies.

4.3.2 DNA metabarcoding

DNA was extracted using a Promega 'Maxwell 16' instrument and a Maxwell® 16 Tissue DNA Purification Kit. Samples were vortexed prior to extraction and ~ 30mg of each sample was used. PCR inhibitor concentrations were reduced in the DNA by mixing this sub-sample in 250uL of STAR buffer (Roche Diagnostics) prior to extraction. Samples were PCR amplified with a universal metazoan primer set that is highly conserved and amplifies a region of the nuclear small subunit ribosomal DNA gene (18S rDNA;McInnes et al. 2017a - Chapter 3). Sequencing of PCR products was performed over two runs with a MiSeq genome sequencer, using the MiSeq V2 reagent kits (300 cycles). DNA extractions, PCR amplification and sequencing followed the methods used in McInnes *et al* (2017a - Chapter 3). A two-stage PCR process was used to enable amplification of the DNA region and attachment of unique 'tag' sequences to each sample which allows amplified samples to be pooled (Binladen *et al.* 2007). Stage one PCR reactions (10 µL) were performed with 5 µL 2x Phusion HF (NEB), 1 µL 100x Bovine Serum Albumin (NEB), 0.1 µL 5uM of each 18S_SSU amplification primer (Table 4.1), 0.5 µl of Evagreen, 2 µL faecal DNA and 1.3 µL of water. Thermal cycling conditions were 98°C, for 2 mins; followed by 35 cycles of 98°C for 5s, 67°C for 20s, 72°C for 20s, with an extension of 72°C for 1 min. Each sample was run in triplicate on a LightCycler 480 (Roche Diagnostics). A negative control containing no template DNA and positive control containing fish DNA were included in each PCR amplification run. If either the negative amplified or the positive failed to amplify, the PCR was

re-run. If ≥2 replicates of each sample had a 'crossing threshold' (ct) score < 30 they were combined to reduce biases produced by amplification from low template concentration samples (Murray, Coghlan & Bunce 2015). Pooled samples were diluted 1:10 for the second stage PCR. A unique tag was attached to each sample in 10 µL PCR reactions with 5 µL 2x Phusion HF (NEB), 1 µL 100x Bovine Serum Albumin (NEB), 1 µL of 1 µM of each tag primer, and 2 µL of diluted PCR product from stage one. Thermal cycling conditions were 98°C, for 2 mins; followed by 10 cycles of 98°C for 5s, 55°C for 20s, 72°C for 20s, with an extension of 72°C for 1 min. Four microlitres of PCR product from each sample and the negative controls were pooled and purified from unincorporated reaction components by washing utilising reversible binding to Agencourt Ampure magnetic beads, with 1.8 µl of Ampure per microlitre of DNA product. Sequencing of PCR products was performed with a MiSeq genome sequencer (Illumina), using the MiSeq reagent kit V2 (300 cycles) with paired-end reads. Samples were split over two sequencing runs. A blocking primer was not used in this study as they may inadvertently block similar groups such as other vertebrates like fish (Piñol et al. 2015). This likely reduced the samples size, but provided more reliable results from higher quality samples containing more food DNA. A breakdown of the proportion of DNA sequences originating from non-food groups for each site can be found in Appendix 4.1.

4.3.3 Bioinformatics

Amplicon pools were de-multiplexed based on unique 10 bp Multiplex IDentifiers (MIDs) incorporated in the Illumina two-step MID protocol using our custom R script (Appendix 4.2). Fastq files were processed using USEARCH v8.0.1623 (Edgar 2010). Reads R1 and R2 from the paired end sequencing were merged using the fastq_mergepairs function, retaining only merged reads flanked by exact matches to the 18S_SSU primers and primer sequences were trimmed. Reads from all samples were pooled and dereplicated using full length matching (-derep_fulllength), then clustered into Operational Taxonomic Units (OTUs) using the cluster_otus command (-otu_radius_pct = 10). Potentially chimeric reads are discarded during this step. Reads for each sample were assigned to these OTUs (usearch_global -id 0.97) and a summary table generated using a custom R script (Appendix 4.2). Each OTU was identified by BLAST and categoried to closest match using MEGAN 5 and the Lowest Common Ancestor (LCA) assignment algorithm (Huson et al. 2007). LCA parameters were set at a minimum score of 250 and a top-percent of 5%. These cut-offs were determined by manually checking a sub-set of samples against BLAST. OTUs from the 18S primers were categorised into food or non-food items based on their taxonomy, so that, for example, obligate parasites and groups highly unlikely to be food such as land plants were 'non-food' (Jarman et al. 2013, McInnes et al. 2017a, Chapter 3 - Appendix 3.2).

4.3.4 Analysis

Samples were included in the final analysis if they contained at least 100 sequences that could be assigned to a food group (Jarman et al. 2013). The diet data were presented using two dietary metrics to reduce any biases caused by reporting one alone. The frequency of occurrence (FOO) was calculated as the total number of samples at each site-year combination containing a given food group. FOO calculations were based on food items which comprised >1% of food sequences for that sample. The second metric used was the proportion of sequences in a sample, or relative read abundance (RRA). This was calculated as the total sequence reads for each prey group divided by the total food sequences in that sample. The mean RRA was calculated for each site-year combination. Both metrics have inherent biases. FOO can overestimate the importance of common prey groups eaten only in small amounts, including secondary ingestion (the food consumed by the prey species). The RRA may not accurately reflect the exact proportion of each prey group consumed, however, has been shown to be representative of the relative diet proportion of prey items in feeding trails (Deagle et al. 2010, Willerslev et al. 2014) and using stable isotope analysis (Kartzinel et al. 2015). The RRA provides a viable option for dietary studies, particularly as a way to distinguish between primary and secondary prey items. To achieve the latter, samples were categorised according to the prey group represented by >70% of the sequences. This enabled assessment of the relative contribution of each prey group. If no major group dominated, the sample was classified as “mixed”. As samples were collected from both chicks and adults, the mixed category could represent an adult feeding on multiple prey groups or a chick fed from different parents.

Statistical analyses were carried out using R software (R Core Team 2015). Poisson generalised linear models (GLM) with a log link function were used to test for differences in prey groups between breeding colonies and years, and between years and breeding stages at each colony. The model included the count of samples (n) as the dependant variable and predictor variables included prey group (P), year (Y) and breeding stage (S), or colony (C). The base model included the sample size as a function of the main effects (prey group, year, breeding stage or colony) as well as the year:stage or year:colony interaction. These terms effectively describe the patterns in the data arising from the experimental sampling process (e.g. total number of samples within a given year). The interaction terms, prey:year, prey:stage or prey:colony were added to the base model to test the effect of year or stage (or colony for the pooled data) on diet composition. The analysis of deviance (with Chi-squared test) and Akaike’s information criterion (AIC) were used to compare fitted models and test the significance of predictor terms (Burnham and Anderson 2002). Dissimilarity indices were

calculated with the Manhattan method using the command 'vegdist' in the package 'Vegan' (Oksanen et al. 2016). From these indices, a hierarchical clustering was then constructed using the average agglomeration method. The command 'simprof' from the package 'clustsig' was used on FOO and RRA data to assess if any significantly different site clusters were present, with a significance of $p < 0.05$ (Whitaker and Christman 2014).

4.3.5 Fishery catch data

It is difficult to determine the availability of prey within the marine environment due to its relative inaccessibility. However, an approximation of jellyfish abundance can be assessed from trawl fishery catch data. Trawl fisheries operate in waters adjacent to the Falkland Islands year-round, where jellyfish are caught as bycatch during fishing operations. Weights of the jellyfish portion of catch are reported daily by captains to the Directorate of Natural Resources – Fisheries of the Falkland Islands Government. Monthly and annual jellyfish catch data were obtained for trawl fishing vessels operating in the Falkland Islands Interim and Outer Conservation Zones (FICZ/FOCZ) between 2011-2016. Data were provided by the Directorate of Natural Resources of the Falkland Islands Government. The total fishing effort (measured in fishing days) and the amount of jellyfish caught per fishing day each month (total tonnes jellyfish/ fishing day) were calculated. There are up to 44 vessels operating in the fishing ground during a given month. Fishing activity is typically low in January and there were no trawl operations in January 2014.

4.4 Results

4.4.1 Amplification success

A total of 1460 scat samples were collected across all islands and years. DNA was amplified in 1039 samples, and 449 samples provided >100 food sequences. The prevalence of non-food DNA (i.e. from the bird, parasites, etc.) in many samples is typical when using "universal" eukaryote PCR primers (McInnes et al. 2017a - Chapter 3). Only two samples from Albatross Islet in 2016 contained food DNA, therefore that year of data was not included, resulting in 447 samples used in subsequent analyses (see Appendix 4.1). Of these samples, 61 were from incubation, 240 from early chick rearing and 146 from late chick-rearing.

4.4.2 Overall diet composition

Actinopterygii (bony fish) were found to be the most abundant prey group overall, present in 86% of samples (FOO) and comprising 66% of food DNA sequences (RRA). Scyphozoa (true jellyfish) were

present in 37% of samples and comprising 20% of food DNA sequences. Other prey items included Crustacea 30% FOO (8% RRA), Cephalopoda 10% FOO (3% RRA), Hydrozoa 6% FOO (2% RRA), Chondrichthyes (skates, sharks, rays) 5% FOO (2% RRA); and Anthozoa, Ctenophora and Tunicata with 2%, 1% and 3% FOO respectively (< 1% RRA; Table 4.1, Figure 4.2 and 4.3).

There was a significant difference in the frequency of occurrence of prey groups detected between years (base model AIC 636.6, P:Y AIC =615.6; $\chi^2_{16} = 53.03$, $p < 0.001$) and breeding colonies (P:C AIC =490.6; $\chi^2_{56} = 258$, $p < 0.001$), however, the inclusion of colony alone provided the best model fit. There was no significant improvement to the model when year and colony were both included (P:Y and P:C AIC =503; $\chi^2_{118} = 258$, $p < 0.276$), which suggests that any year differences were likely to be an artefact of different colonies sampled. Although there was some variation in the prey detected between breeding stages (Appendix 4.3), there was no significant effect of breeding stage or year on the frequency of prey groups detected when each colony was tested individually. At each site the base model provided the best fit of the data (see Appendix 4.4).

When each sample was classified according to the dominant prey group (> 70% of sequences), samples fell into the six main prey groups listed above. Anthozoa, Ctenophora and Tunicata were present in samples in low proportions, and always co-occurred with other prey items, suggesting they may have represented secondary ingestion. A small percentage of samples (10%) were classified as 'mixed' (Figure 4.3). Using FOO data, there was no significant site clusters identified, whereas using RRA, sites were clustered into two significantly different groups ($p < 0.05$; Figure 4.4). Cluster 1 included Campbell, Steeple Jason and Macquarie islands, and cluster 2 included the remainder. The main differences were the ratio of Actinopterygii to Scyphozoa. In both clusters these two prey groups together contributed 85% of the sequences; however, in group 1 the ratio of Actinopterygii to Scyphozoa was 1.3 : 1 compared to 13.7 : 1 for group 2 (Figure 4.4).

Cnidarians

Cnidarian DNA occurred in a large proportion of samples and comprised a high proportion of sequence reads at several black-browed albatross sites and at Campbell Island (Fig 2, Table 4.1). Scyphozoan jellyfish from the orders Coronatae (crown jellyfish) and Semaeostomeae were the main gelatinous prey DNA detected. The occurrence of these two orders differed between sites; Semaeostomeae were detected at all sites, although only in large proportions at Steeple Jason Island (53 – 78% FOO, 30 – 50% RRA) and Campbell Island (23 – 44% FOO, 13 – 21% RRA), whereas Coronatae was detected mostly at Macquarie Island (50 – 64% FOO; 35 – 41% RRA; Table 4.1).

Hydrozoans from the order Siphonophorae were found in albatross scat samples from Campbell Island in 2014 (31.1% FOO, 17.5% RRA). Anthozoa from the order Actiniaria (sea anemone) occurred in relatively low proportions, the highest in samples from Campbell Island in 2015 (7.7% FOO and 6.9% RRA); all co-occurred with Semaeostomeae.

Crustaceans

Crustacean DNA occurred in greater than 10% of samples at each site, however constituted less than 5% of prey sequences at most sites. The exceptions to the latter were New Island in both years (60 – 68% FOO, 20 – 24% RRA), Campbell Island in 2015 (35 – 46% FOO, 6 – 23% RRA), Bird Island in 2014 (12% FOO, 7% RRA) and Diego Ramírez in 2014 (21% FOO, 12% RRA). Crustaceans in the diet at New Island were mostly from the family Munidae (lobster krill), at Campbell Island from the sub-order Lepadomorpha (goose barnacles), at Bird Island from the order Euphausiacea (krill) and at Diego Ramírez from the order Podoplea, although the latter all co-occurred with fish and so may represent secondary ingestion.

Cephalopoda

Cephalopoda DNA occurred in greater than 5% of samples at all sites, and greater than 10% of samples at Macquarie Island, Steeple Jason Island, Bird Island (2015), Diego Ramirez and Campbell Island (2014). However, at only three sites was the RRA of cephalopod sequences > 5%. These were Bird Island in 2015 (14% FOO, 8% RRA), Steeple Jason Island in 2015 (15% FOO, 8% RRA) and Diego Ramírez in 2014 (11% FOO, 5% RRA) and in each case, were almost all from the order Teuthida (squids).

4.4.3 Jellyfish abundance at the Falkland Islands

Between 2011 and 2016, there have been variable amounts of jellyfish caught in the trawl fishery at the Falkland Islands, with large jellyfish blooms evident in 2014 and 2016 (Figure 4.5). There is also a seasonal pattern of abundance evident with higher jellyfish catches per fishing day from February – April, which overlaps temporally with the albatross chick rearing period. There was no fishing activity in January 2014, therefore no jellyfish catch data. The two seasons that albatross diet sampling occurred corresponded with a year of high jellyfish catch reported in 2014 (~ 3800 tonnes) and a year of low catch reported in 2015 (~330 tonnes; Figure 4.5), with a ten-fold difference in reported catch between years. Overall, there was no statistical difference in albatross diets between years at each site, or between breeding stages (Appendix 4.4). However, at Steeple Jason Island a higher proportion of samples contained jellyfish DNA during late chick-rearing (80-100% FOO) compared to

incubation (40% FOO) and early chick-rearing (20-56% FOO; Figure 4.5, Appendix 4.3). Even though there was large differences in the fishery catch in March of each year, this was not reflected in the diet (100% FOO, 60% RRA in March 2014 and 88% FOO and 53% RRA in March 2015). The breeding success at New Island and Steeple Jason were similarly high in both years of the study, irrespective of higher prevalence of jellyfish in the diet at Steeple Jason (Breeding success: New Island 84.3% and 80.8% and Steeple Jason 60.1 and 81.8% in 2014 and 2015 respectively).

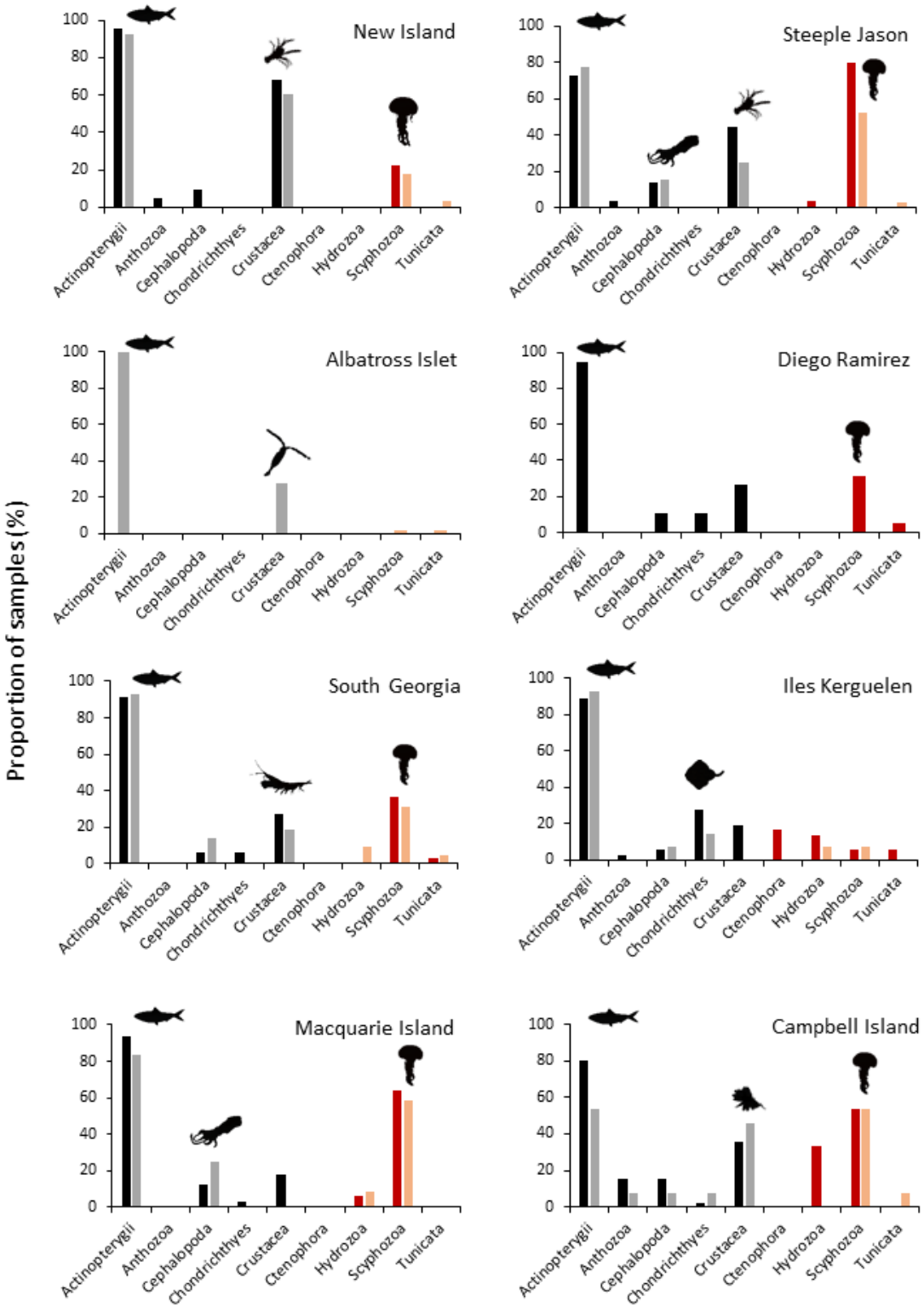

Figure 4.2 The frequency of occurrence of prey groups in the diet of black-browed and Campbell albatrosses from austral summer 2013/14-2015/16. Dark bars represent 2013/14 collections and the lighter bar 2014/15 (or in the case of Iles Kerguelen, 2015/16). The red and orange bars highlight the gelatinous prey items detected.

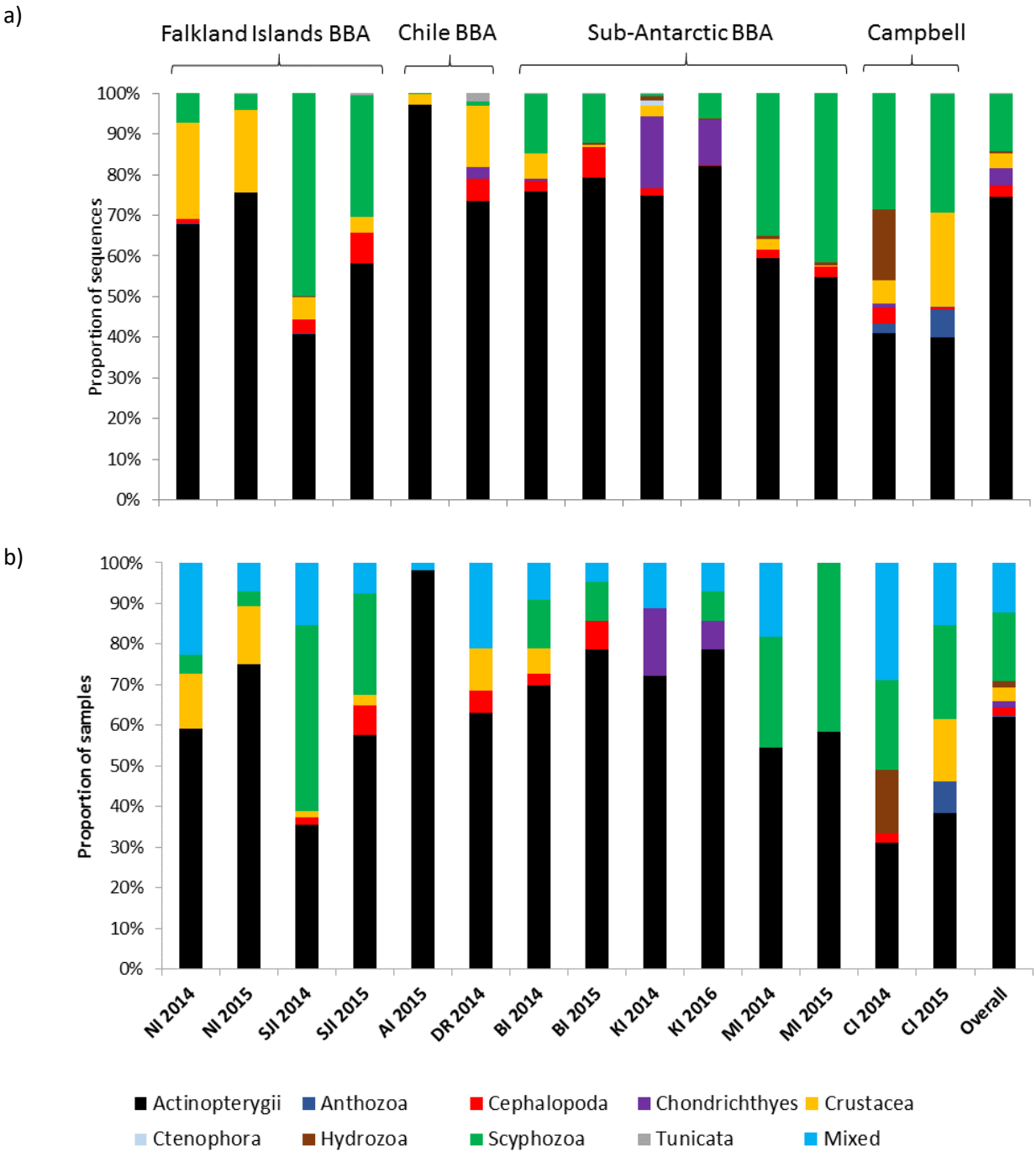

Figure 4.3 The relative read abundance and major prey groups consumed by black-browed and Campbell Island albatrosses from austral summer 2013/14-2015/16. Values represent (a) relative read abundance for each site and year and (b) the proportion of samples with >70% of sequences assigned to each prey group. Mixed samples have <70% of the sequences from any one group. Sites were: New Island (NI) and Steeple Jason Island (SJI), Falkland Islands; Diego Ramírez (DR) and Albatross Islet (AI), Chile; Bird Island, South Georgia (BI); Kerguelen Archipelago (KI), France; Macquarie Island, Australia (MI); and Campbell Island, NZ (CI).

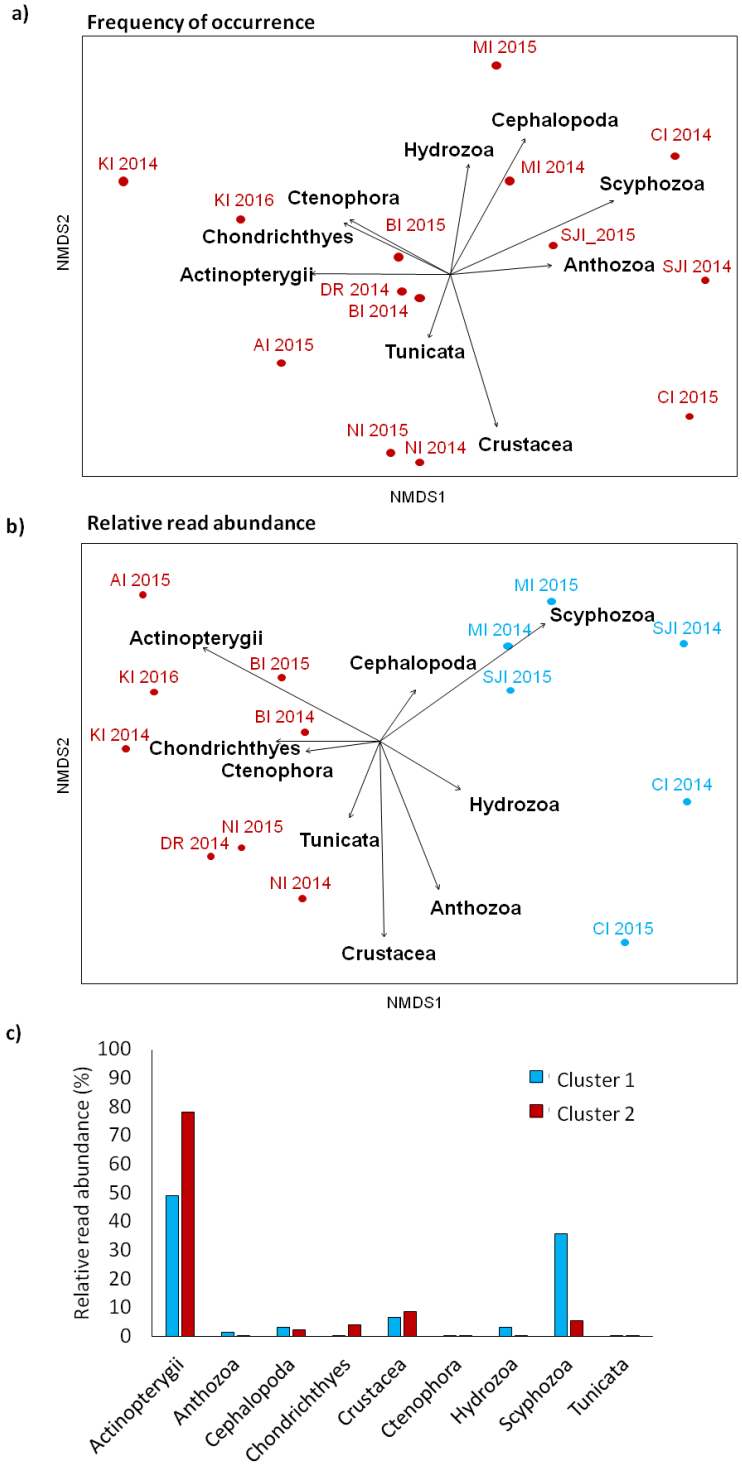

Figure 4.4 Correspondence of breeding sites with prevalence of major prey groups indicated by multi-dimensional scaling using: a) frequency of occurrence (FOO) and b) relative read abundance (RRA). Significantly different site clusters are shown in red and blue in figure b and the RRA for each group in figure C. The mean RRA of prey sequences for each group are shown in the bar plot with the ratio of Actinopterygii (bony fish) to Scyphozoa (jellyfish) resulting in the major division. Clusters were assigned using dissimilarity indices calculated with the Manhattan method and hierarchical clustering was calculated using the average agglomeration method.

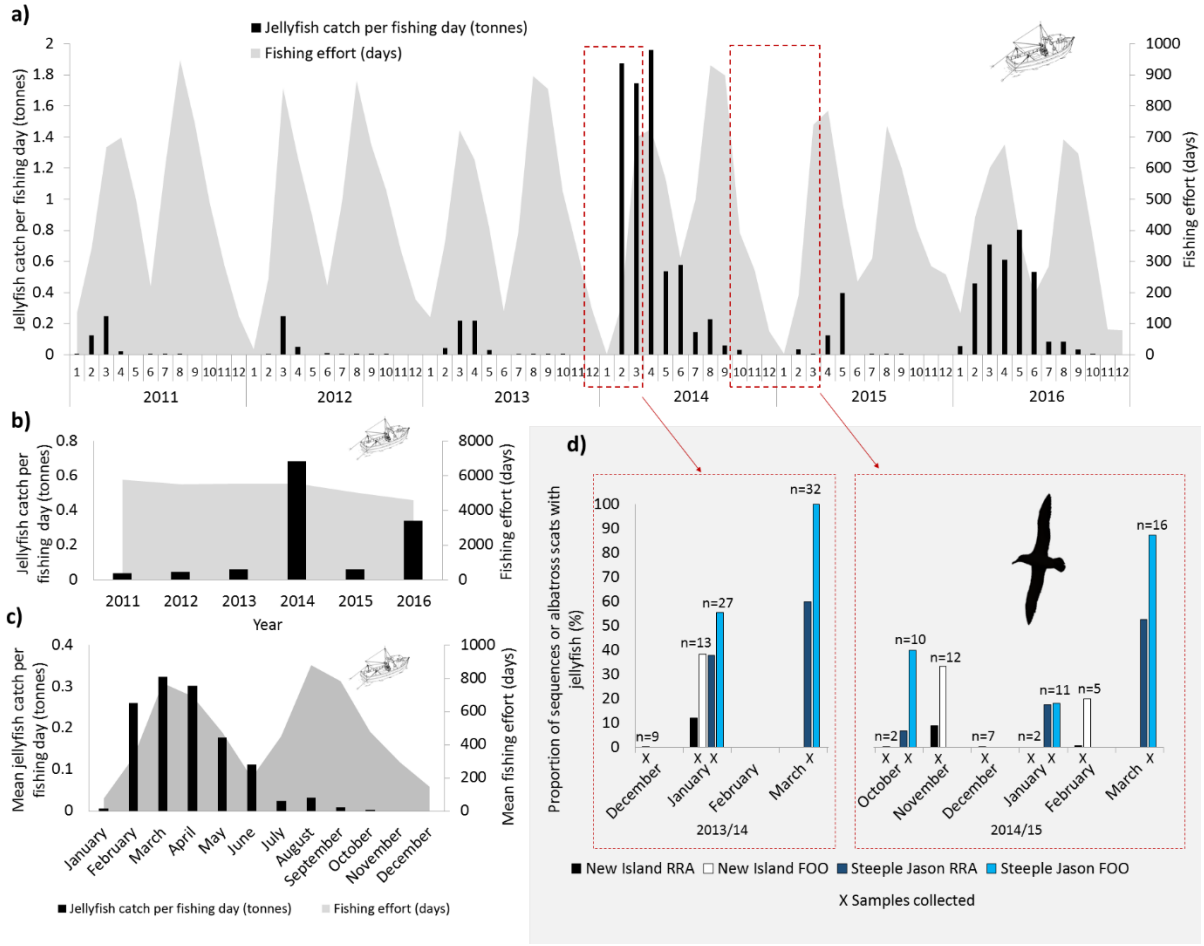

Figure 4.5 The amount of jellyfish caught in trawl fisheries off the Falkland Islands from 2011-2016 and amount of jellyfish in the diet of black-browed albatross during this study. Grey shading represents the trawl fishing effort in days and the black bars show the amount of jellyfish caught in tonnes per fishing day across: a) each month from 2011-2016, b) each year from 2011-2016 and c) average monthly totals. Figure d) shows the average monthly RRA and FOO of jellyfish DNA in albatross scat samples at New Island and Steeple Jason Island. The 'x' represents sampling periods for each site to distinguish between no jellyfish detection and no dietary sampling.

4.5 Discussion

This is the first study of albatross diets covering the same or sibling species at such a wide geographic scale and employing synchronous sampling at multiple sites. Our results confirm the hypothesis that gelatinous prey, specifically scyphozoan jellyfish (hereafter termed jellyfish), are a common prey of black-browed and Campbell albatross. We also show that the frequency of jellyfish occurrence in the diet was similar in years of high and low relative jellyfish abundance, suggesting that consumption is not purely opportunistic.

Our hypothesis that gelatinous animals are a common prey item in albatross diet was motivated by the apparent discrepancy between at-sea observations of albatross foraging on jellyfish, yet low detection rates in stomach contents (11% of black browed studies and < 5% of meal mass when present). Additionally, previous DNA metabarcoding of penguin scats has identified frequent occurrence of jellyfish in the diets which has not been detected often using stomach content analyses (Jarman et al. 2013, McInnes et al. 2016a). We found an even higher frequency of occurrence of gelatinous prey in albatross diets than the penguin studies and much higher than studies on albatross using conventional methods. Jellyfish were present at seven of the eight sampled breeding sites and were a common prey item at three of these sites with up to 80% of samples from Steeple Jason Island containing jellyfish and comprising 50% of DNA sequences. hydrozoans, anthozoans and ctenophores were also detected during this study, though with the latter two in low proportions.

High rates of jellyfish ingestion have not been detected in previous albatross studies (Cherel and Klages 1998), which is likely explained by the limitations of stomach content analyses. Predation on jellyfish by black-browed albatross has been observed previously at sea (Weimerskirch et al. 1986, Suazo 2008) and at Beauchêne Island in the Falkland Islands where jellyfish were found in 20% of samples (< 10% mass; Thompson 1992). Our suggestion that they may be consumed during self-feeding rather than provisioning was not supported, with jellyfish detected in the diets of chicks during late chick-rearing and adults during incubation and early-chick rearing. This finding provides further evidence that low detection rates reported in previous studies were not purely the result of sampling timing.

The frequency of jellyfish occurrence varied extensively between colonies. Almost no jellyfish were found in the diet at the Chilean sites or at Kerguelen in 2014. Although at Diego Ramirez jellyfish

occurred in 30% of samples, the RRA was only 1% and no samples contained jellyfish as the main prey item (Figure 4.3b). When sites were clustered into groups by diet, the main difference was the RRA of fish and jellyfish sequences. This division had no relationship with site proximity suggesting this is not just a localised occurrence. Albatross at Steeple Jason and New Island had very different diets in both years and clustered into separate groups, even though these sites are only 70km apart. This is consistent with previous dietary work at the Falkland Islands that found large differences between colonies (Thompson 1992), suggesting birds from the two colonies use distinct foraging grounds, which has been confirmed by subsequent tracking studies (Catry et al. 2013). Across all sites, spatial differences in diet were greater than temporal differences, suggesting that the site-by-site differences relate to site-specific factors such as local prey abundance or learned foraging preferences. However, jellyfish availability estimates from the Falkland Islands indicate that the consumption of jellyfish is not based purely on availability of prey. During this study, the frequency of jellyfish occurrence in the scats of black-browed albatross was similar in years of high and low availability, which suggests that they may actively be targeting jellyfish.

Seabirds have been found to target jellyfish aggregations to forage on juvenile fish that associate with jellyfish for food or protection (Sato et al. 2015), and therefore consumption of jellyfish could be accidental or secondary in such cases where jellyfish are predated by fish (Milisenda et al. 2014). DNA metabarcoding can detect DNA from secondary ingestion (Jarman et al. 2013). In our study, the detection of anthozoans, ctenophores and tunicates were likely to be through secondary ingestion as they occurred only in low abundance and always co-occurred with other prey items. However, this was unlikely to be the case for hydrozoans and Scyphozoan jellyfish. The FOO and RRA from both prey groups were high for sites where they were detected regularly, whereas we would expect the RRA to be much lower if predation was secondary. When these hydrozoans and scyphozoans were consumed by an individual, they were often the dominant item (> 70% RRA). This was further confirmed by some samples where they were the only food DNA present in the sample.

During this study, the breeding success was similarly high at Steeple Jason and New Island in 2015 even though jellyfish occurred more frequently in the diet at Steeple Jason Island. The breeding success was slightly lower at Steeple Jason in 2014, however, was still higher than the long-term average at the Falkland Islands (New Island 56% from 2004-2009), and higher than conspecifics at other island groups (ACAP 2010, Catry et al. 2011). This suggests that the consumption of jellyfish by albatross may not be impacting breeding success at the population level. However, the consequences of choosing this prey at the individual level and the effect on chick fledging mass is

unknown. An increase in easily accessible but energetically poor food may be a good short-term solution when higher energy prey is scarce, but over the long-term the impacts of low nutritional prey in albatross diets are unknown. For other marine predators low nutritional prey has reduced body condition, breeding success, and ultimately survival (Rosen and Trites 2000, Kitaysky et al. 2006, Grémillet et al. 2008).

A challenge of seabird dietary studies is the inability to accurately quantify the available biomass of potential prey species. The majority of marine ecosystem monitoring studies measure from the top-down rather than bottom-up, which makes it difficult to determine the reasons for prey selection. This is especially the case in the Southern Ocean, which is one of the most inaccessible places on earth. The Falkland Islands in the South Atlantic provided a unique opportunity to gain an insight into the relative occurrence of jellyfish across multiple years through catch data. These catch amounts do not provide a definitive biomass of jellyfish, but instead give an indication of relative jellyfish prevalence in the sea across years. There are several factors that should be considered when interpreting this data. Jellyfish are caught as bycatch rather than targeted by the fishery and are likely to be actively avoided where possible, including making shorter trawls to avoid damage to fishing gear (FIG 2015). The jellyfish catch data in this study is also over a broad scale around the Falkland Islands rather than specifically relating to the albatross foraging area, therefore does not allow interpretation of fine-scale changes in jellyfish abundance. However, the ten-fold increase in the jellyfish catch in 2014 from 2013 and then back to similarly low levels in 2015, is large enough to give an indication of major differences in the jellyfish prevalence between years. More in-depth studies using finer-scale jellyfish biomass estimates around both New Island and Steeple Jason colonies would provide a more robust estimate of jellyfish abundance and albatross prey choices. This could also be studied across multiple breeding stages. Although there was no statistical difference between breeding stages at Steeple Jason, there was a trend for a higher occurrence of jellyfish in the diet during late chick-rearing (80-100%) compared to incubation (40%) and early chick-rearing (20-56%) and a similar trend at Macquarie Island and Bird Island (Appendix 4.3). This pattern is consistent with a switch to low-energy prey late in the breeding season when high nutrient food near colonies can be depleted (Ashmole 1963). Black-browed albatross are known to consistently return to the same foraging sites (Weimerskirch et al. 1986), therefore the ability to switch prey would be advantageous as it allows for more flexibility, especially when resources are scarce.

Ongoing monitoring of diet and foraging ecology of top predators will help characterise the impacts of environmental change and fisheries on breeding populations (Furness 1982, Croxall et al. 1999, Constable 2001). Climate change is predicted to cause major changes in the abundance and distribution of marine species (Constable et al. 2014). Jellyfish typically benefit from perturbations to the marine environment (Purcell 2012), such as ocean warming (Purcell 2005, Quiñones et al. 2015) overfishing (Daskalov et al. 2007), and the increasing number of coastal anthropogenic structures which promote the settlement of early larval stages (Duarte et al. 2013). Population increases are therefore predicted under current climate change scenarios and global trends show a slight increase over the long-term, but show significant oscillations in blooms over shorter time scales (Condon et al. 2013). At the Falkland Islands, there have been two obvious jellyfish blooms in the last six years, however, long-term data are not available. Continued collection of jellyfish catch data would be valuable to understand if these blooms are increasing in prevalence and what impacts this may have on seabird diet.

Cephalopods were only a minor prey item for both species in this study with DNA present in 10% of samples and comprising 2.8% of sequences overall. Although there was up to 50% FOO at Macquarie Island (5% RRA) and 27% FOO (20% RRA) at Steeple Jason Island during early-chick rearing 2015, the overall contribution in this study was much lower than previous stomach content studies. Low squid occurrence has also been inferred from stable isotope analysis of black-browed albatross (Granadeiro et al. 2013), however, cephalopods are typically detected in high proportions in stomach contents. Previous black-browed albatross diet studies report that cephalopods occur in 50% of samples on average and 27% of the diet by mass (see Appendix 4.5), although this varied among sites (Thompson 1992, Cherel et al. 2000b) and between years (Arata and Xavier 2003, Xavier et al. 2003a). Although a large number of samples were collected earlier in our study (during incubation and early chick-rearing) than previous studies, cephalopod occurrence was still low in our study during late chick-rearing. Cephalopods do undertake vertical and horizontal migrations during their lifecycles (Arkhipkin et al. 2004), so temporal changes in their availability should be expected. Indeed there have been years where less than 10% of samples from these sites contained cephalopods (Cherel et al. 2000b, Arata and Xavier 2003). Nevertheless, consistently low cephalopod abundance using such a large-scale sampling scheme is unusual, especially as globally, cephalopod populations are increasing (Doubleday et al. 2016).

The low rate of cephalopod DNA in the scats that we observed is unlikely to be the result of a major technical bias in our DNA metabarcoding system. Target sequences from all prey groups were

aligned and checked for primer mismatches or any insertions/deletions that may have affected DNA amplification, none were detected. There is also no evidence from other DNA metabarcoding studies that cephalopod DNA degrades more during digestion than fish DNA, with equal detection of DNA from both prey groups in scats during feeding trials even when squid was a small proportion of the diet (Deagle et al. 2005, Casper et al. 2007). We tested the marker used in our study on both pure squid material and faecal DNA extracts from other albatross and penguin species to ensure that the PCR could detect cephalopod DNA. These tests revealed up to 50% occurrence of cephalopod DNA in scat samples of two other seabird species (unpublished data). Furthermore, to reduce the impact of technical biases, we analysed both the RRA and FOO across samples. The latter analyses will detect cephalopod ingestion even if there is a bias against amplification of their DNA relative to other prey groups. The overall conclusions of theses analyses are similar. More broadly, the reason for these differences between our study using DNA and those using stomach contents is inconclusive. We cannot determine if this simply reflects technical biases introduced by different methods of diet determination or whether cephalopod predation rates were generally lower than previously reported. It would be good to test this observation in future studies with more samples collected in different years, simultaneous stomach content collections or an alternate DNA metabarcoding system which includes group specific markers.

DNA metabarcoding provides a useful new way to study the diet of seabirds. Our study demonstrates that it could enhance long-term ecological monitoring studies to enable all prey groups to be detected. This is particularly important where seabirds used as indicator species (Cairns 1987). For example, in the Southern Ocean an international program uses diet analyses of stomach contents from marine predators as biological indicators of ecosystem health (CEMP; SC-CCAMLR 1997). Two of the key predators studied are black-browed albatross and Adélie penguins *(Pygoscelis adeliae*), both of which have been now been shown using metabarcoding to consume substantial amounts of jellyfish. If there are shifts away from krill towards more gelatinous species in the Southern Ocean (Atkinson et al. 2004), the consequent impacts on predator diets are likely *to be difficult to detect using current methods.* If the biomass of jellyfish increases and/or their distribution shifts, it will be important to identify changes to the food-webs and monitor the short and long-term effects that an abundance of a low nutritional food may have on the body condition, breeding success and survival prospects of marine predators.

4.6 Acknowledgements

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Table 4.1 Prey groups consumed by black-browed albatross at each site and Campbell albatross at Campbell Island in each year. Values represent the frequency of occurrence (FOO) with relative read abundance (RRA) in parenthesis. FOO calculations were calculated for any food item which comprised >1% of food sequences for that sample.

	NI 2014	NI 2015	SJI 2014	SJI 2015	AI 2015	DR 2014	BI 2014	BI 2015	KI 2014	KI 2016	MI 2014	MI 2015	CI 2014	CI 2015	Overall
Sample size	**22**	**28**	**59**	**40**	**51**	**19**	**33**	**42**	**36**	**14**	**33**	**12**	**45**	**13**	**447**
CHORDATA	**95.5 (67.9)**	**92.9 (75.8)**	**72.9 (40.8)**	**77.5 (58.5)**	**100 (97.2)**	**100 (78.6)**	**90.9 (76.6)**	**92.9 (79.5)**	**100 (92.6)**	**100 (93.4)**	**93.9 (59.4)**	**83.3 (54.9)**	**80.0 (42.1)**	**53.8 (40.1)**	**88.1 (68.4)**
Actinopterygii	**95.5 (67.9)**	**92.9 (75.7)**	**72.9 (40.8)**	**77.5 (58.2)**	**100 (97.2)**	**94.7 (73.6)**	**90.9 (75.9)**	**92.9 (79.3)**	**88.9 (74.8)**	**92.9 (82.1)**	**93.9 (59.4)**	**83.3 (54.9)**	**80 (41.1)**	**53.8 (39.9)**	**86.4 (65.8)**
Clupeocephala	95.5 (67.9)	92.9 (75.7)	72.9 (40.7)	77.5 (58.2)	100 (97.2)	94.7 (73.6)	90.9 (75.9)	92.9 (79.3)	88.9 (74.8)	92.9 (82.1)	93.9 (59.4)	83.3 (54.9)	80 (41.1)	53.8 (39.9)	86.4 (65.8)
Chondrichthyes						**10.5 (3)**	**6.1 (0.5)**		**27.8 (17.6)**	**14.3 (11.3)**	**3 (<0.1)**		**2.2 (1)**	**7.7 (0.1)**	**5.1 (2.4)**
Batoidea						10.5 (3)	6.1 (0.5)		27.8 (17.6)	14.3 (11.3)	3 (<0.1)			7.7 (0.1)	5 (2.3)
Selachimorpha													2.2 (0.9)		0.2 (0.1)
Tunicata		**3.6 (0.1)**		**2.5 (0.4)**	**2 (<0.1)**	**5.3 (2.1)**	**3 (0.1)**	**4.8 (0.2)**	**5.6 (0.1)**					**7.7 (0.1)**	**2.5 (0.2)**
CNIDARIA	**31.8 (7.3)**	**17.9 (4)**	**79.7 (50.4)**	**52.5 (30.1)**	**2 (0.1)**	**31.6 (1.1)**	**36.4 (14.7)**	**35.7 (12.5)**	**22.2 (1.7)**	**14.3 (6.4)**	**72.7 (35.9)**	**58.3 (42.5)**	**77.8 (48.3)**	**53.8 (36.1)**	**41.9 (20.8)**
Anthozoa	**4.5 (0.1)**		**3.4 (0.2)**						**2.8 (<0.1)**				**15.6 (2.3)**	**7.7 (6.9)**	**2.4 (0.7)**
Actiniaria	4.5 (0.1)		3.4 (0.2)						2.8 (<0.1)				15.6 (2.3)	7.7 (6.9)	2.4 (0.7)
Hydrozoa	**0 (0.1)**		**3.4 (0.4)**					**9.5 (0.6)**	**13.9 (1)**	**7.1 (0.2)**	**6.1 (0.8)**	**8.3 (0.8)**	**33.3 (17.5)**		**5.8 (1.5)**
Anthomedusae	0 (0.1)		3.4 (0.4)					9.5 (0.6)	13.9 (1)	7.1 (0.2)	6.1 (0.8)	8.3 (0.8)			3.5 (0.3)
Siphonophorae													31.1 (17.5)		2.2 (1.3)
Undetermined													2.2 (<0.1)		0.2 (<0.1)
Scyphozoa	**22.7 (7.1)**	**17.9 (4)**	**79.7 (49.9)**	**52.5 (30.1)**	**2 (0.1)**	**31.6 (1)**	**36.4 (14.6)**	**31 (11.9)**	**5.6 (0.6)**	**7.1 (6.2)**	**63.6 (35.1)**	**58.3 (41.7)**	**53.3 (28.5)**	**53.8 (29.2)**	**36.8 (19.8)**

Coronatae			1.7 (0.2)				24.2 (12.9)	7.1 (2.7)	5.6 (0.6)		63.6 (35.1)	50 (41.1)	44.4 (21.1)	23.1 (12.5)	15.7 (7.5)
Semaeostomeae	22.7 (7.1)	17.9 (4)	78 (49.7)	52.5 (30.1)	2 (0.1)	31.6 (1)	12.1 (1.7)	23.8 (9.1)		7.1 (6.2)		8.3 (0.6)	20 (7.3)	30.8 (16.7)	21.9 (12.3)
Undetermined							3 (<0.1)						2.2 (<0.1)		0.4 (<0.1)
CTENOPHORA															
Ctenophora									**16.7 (1.3)**						**1.2 (0.1)**
MOLLUSCA															
Cephalopoda	**9.1 (1.1)**		**13.6 (3.4)**	**15 (7.6)**		**10.5 (5.3)**	**6.1 (2.6)**	**14.3 (7.5)**	**5.6 (1.8)**	**7.1 (0.2)**	**12.1 (2)**	**25 (2.6)**	**15.6 (3.9)**	**7.7 (0.6)**	**10.1 (2.8)**
Octopoda			1.7 (0.1)						2.8 (0.6)						0.3 (0.1)
Teuthida	4.5 (0.2)		13.6 (3.3)	15 (7.6)		5.3 (5.2)	6.1 (2.6)	14.3 (7.5)	2.8 (1.2)	7.1 (0.2)	9.1 (1.9)	8.3 (0.3)	13.3 (3.8)	7.7 (0.6)	7.7 (2.5)
Unidentified	4.5 (0.9)					5.3 (0.1)					3 (0.1)	16.7 (2.3)	6.7 (0.2)		2.6 (0.3)
ANTHROPODA															
Crustacea	**68.2 (23.6)**	**60.7 (20.1)**	**44.1 (5.4)**	**25 (3.7)**	**27.5 (2.7)**	**26.3 (15)**	**27.3 (6.1)**	**19 (0.5)**	**19.4 (2.6)**		**18.2 (2.6)**		**35.6 (5.7)**	**46.2 (23.2)**	**29.8 (7.9)**
Calanoida	40.9 (4.5)	14.3 (3.6)	28.8 (2.4)	5 (0.2)	23.5 (2)	10.5 (3.2)	3 (0.1)	7.1 (0.2)	13.9 (1.2)		6.1 (0.2)		20 (3.7)	15.4 (0.7)	13.5 (1.6)
Decapoda													2.2 (0.1)		0.2 (<0.1)
Eumalacostraca	4.5 (0.8)	28.6 (5)	8.5 (0.3)	12.5 (2.9)			6.1 (0.2)		2.8 (0.1)		3 (0.1)			7.7 (0.3)	5.3 (0.7)
Euphausiacea	4.5 (0.1)	7.1 (0.4)		12.5 (0.5)			18.2 (5.7)	7.1 (0.3)						0 (0.1)	3.5 (0.5)
Isopoda			1.7 (1.6)	2.5 (0)					2.8 (1.1)						0.5 (0.2)

Munididae	27.3 (15.7)	21.4 (11)	5.1 (1)										3.8 (2)
Peracarida							2.4 (<0.1)		3 (0.1)		2.2 (0.1)	15.4 (0.2)	1.6 (<0.1)
Pleocyemata	9.1 (2.4)	3.6 (0.1)										7.7 (0.3)	1.5 (0.2)
Podoplea	4.5 (0.1)			9.8 (0.6)	21.1 (11.7)			2.8 (0.1)	6.1 (2.2)		13.3 (1.7)		4.1 (1.2)
Thoracica											4.4 (0.2)	30.8 (21.6)	2.5 (1.6)
Undetermined			1.7 (0.1)			3 (<0.1)							0.3 (<0.1)

4.8 Appendices

Appendix 4.1.1: DNA amplification and sample size total

	Year	# Collected	18s Amplified	Amplification success	Food	Aves	Contaminant	Ecto-parasites	Endo-parasites	Fungi	Mammal	Plant	Uni-cellular	Un-matched	>100 Sequences Food	Prop with >100 seq food
Albatross Islet	2015	85	64	75.3	61.8	20.8	0.2	0.0	6.0	2.8	0.5	3.2	1.4	3.1	51	60.0
	2016	19	19	100.0	4.0	24.2	0.1	5.2	23.2	24.5	5.5	3.0	3.5	6.6	2	10.5
Bird Island	2014	150	102	68.0	13.4	18.8	0.3	4.0	6.7	7.1	0.4	33.8	8.6	6.9	33	22.0
	2015	107	77	72.0	33.3	23.0	0.1	1.8	7.8	12.1	0.1	12.3	1.9	7.6	42	39.3
Campbell Island	2014	195	119	61.0	11.7	56.8	0.3	1.0	2.8	9.1	0.1	6.2	4.2	7.7	45	23.1
	2015	54	49	90.7	21.7	25.6	0.8	0.1	6.1	6.4	1.2	4.3	16.2	17.7	13	24.1
Diego Ramirez	2014	99	59	59.6	3.6	33.6	6.2	0.1	4.3	6.1	0.1	13.4	19.3	13.3	19	19.2
Kerguelen	2014	82	53	64.6	44.4	27.4	0.1	4.3	11.0	2.9	0.0	6.4	1.5	1.9	36	43.9
	2016	26	25	96.2	46.3	21.7	1.6	4.8	8.8	6.2	0.2	7.2	0.7	2.4	14	53.8
Macquarie Island	2014	73	50	68.5	37.9	23.1	2.9	1.0	4.2	11.2	0.1	10.9	3.3	5.4	33	45.2
	2015	49	43	87.8	20.6	28.9	1.1	3.4	6.7	8.9	0.1	20.5	4.0	5.8	12	24.5
New Island	2014	63	48	76.2	21.1	49.7	0.1	0.0	16.4	2.0	0.1	2.4	2.5	5.7	22	34.9
	2015	127	104	81.9	22.2	30.3	0.4	1.4	27.7	4.6	0.6	4.8	3.3	4.9	28	22.0
Steeple Jason	2014	133	91	68.4	28.3	30.6	1.1	0.4	21.6	3.3	0.1	3.3	6.0	5.2	59	44.4
	2015	198	136	68.7	19.1	43.2	0.1	0.1	10.1	8.6	0.8	7.0	2.7	8.2	40	20.2
		1460	**1039**	**71.2**	**24.5**	**32.8**	**0.3**	**1.5**	**10.9**	**7.1**	**0.5**	**9.9**	**5.2**	**6.9**	**449**	**30.8**

Appendix 4.1.2: Sample size per breeding stage

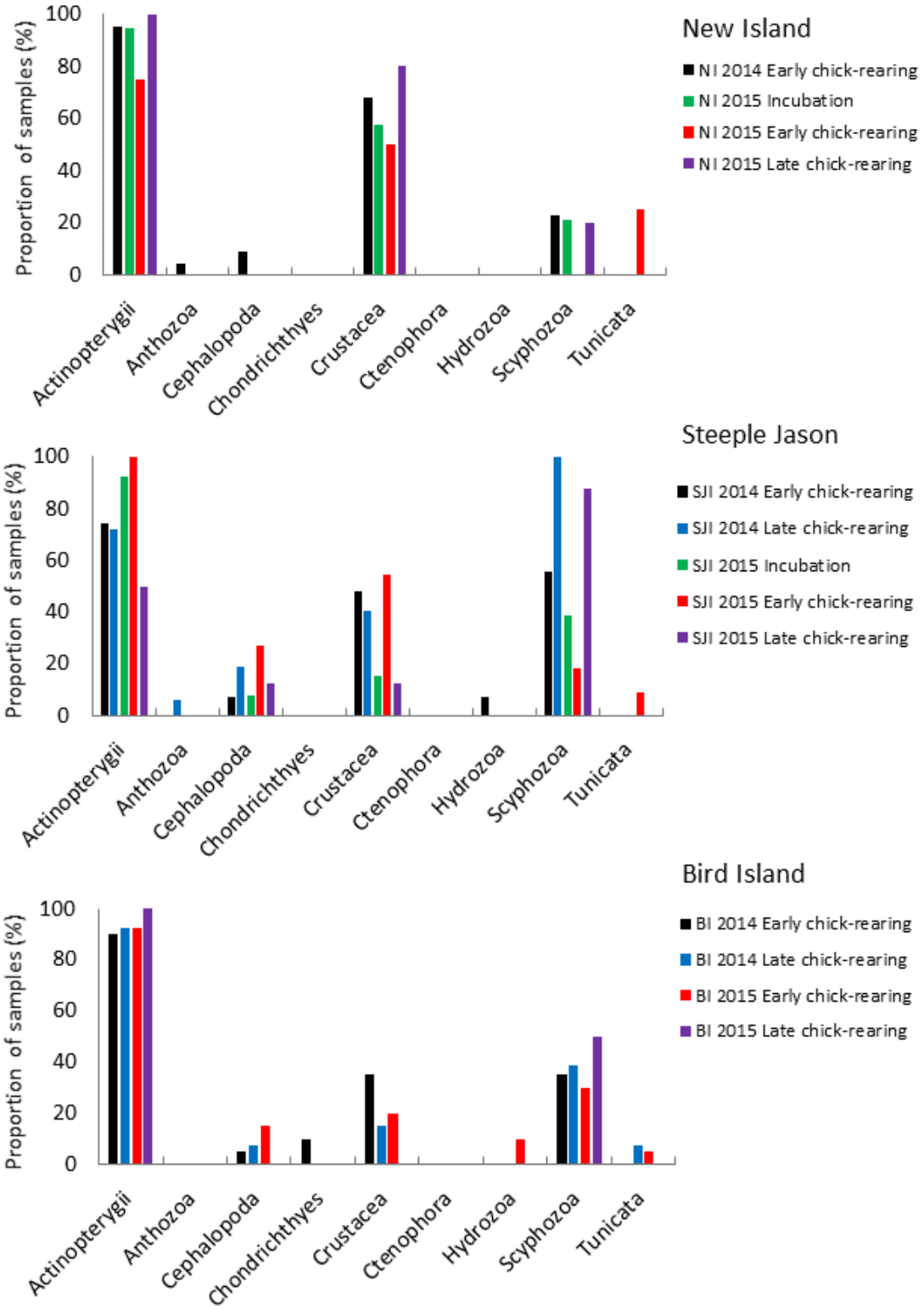
		# Collected			DNA Amplified			>100 food seqences			DNA amplification success			Proportion with food DNA		
	Year	Incubation	Early-chick rearing	Late-chick rearing	Incubation	Early-chick rearing	Late-chick rearing	Incubation	Early-chick rearing	Late-chick rearing	Incubation	Early-chick rearing	Late-chick rearing	Incubation	Early-chick rearing	Late-chick rearing
Albatross Islet	2015			85			64			**51**			75.3			60.0
	2016			19			19			**2**			100.0			10.5
Bird Island	2014		89	61		61	41		**20**	**13**		68.5	67.2		22.5	21.3
	2015		79	28		65	12		**40**	**2**		82.3	42.9		50.6	7.1
Campbell Island	2014	95	100		55	64		**14**	**31**		57.9	64.0		14.7	31.0	
	2015		54			49			**13**			90.7			24.1	
Diego Ramirez	2014			99			59			**19**			59.6			19.2
Kerguelen	2014	29	53		15	38		**9**	**27**		51.7	71.7		31.0	50.9	
	2016		26			25			**14**			96.2			53.8	
Macquarie Island	2014	11	37	25	7	25	18	**7**	**18**	**8**	63.6	67.6	72.0	63.6	48.6	32.0
	2015	19	13	17	16	11	16	**4**	**8**		84.2	84.6	94.1	21.1	61.5	0.0
New Island	2014		63			48			**22**			76.2			34.9	
	2015	83	33	11	71	24	9	**14**	**9**	**5**	85.5	72.7	81.8	16.9	27.3	45.5
Steeple Jason	2014		76	57		47	44		**27**	**32**		61.8	77.2		35.5	56.1
	2015	38	95	65	29	63	44	**13**	**11**	**16**	76.3	66.3	67.7	34.2	11.6	24.6
		275	**718**	**467**	**193**	**520**	**326**	**61**	**240**	**148**	69.9	75.2	73.8	30.3	37.7	27.6

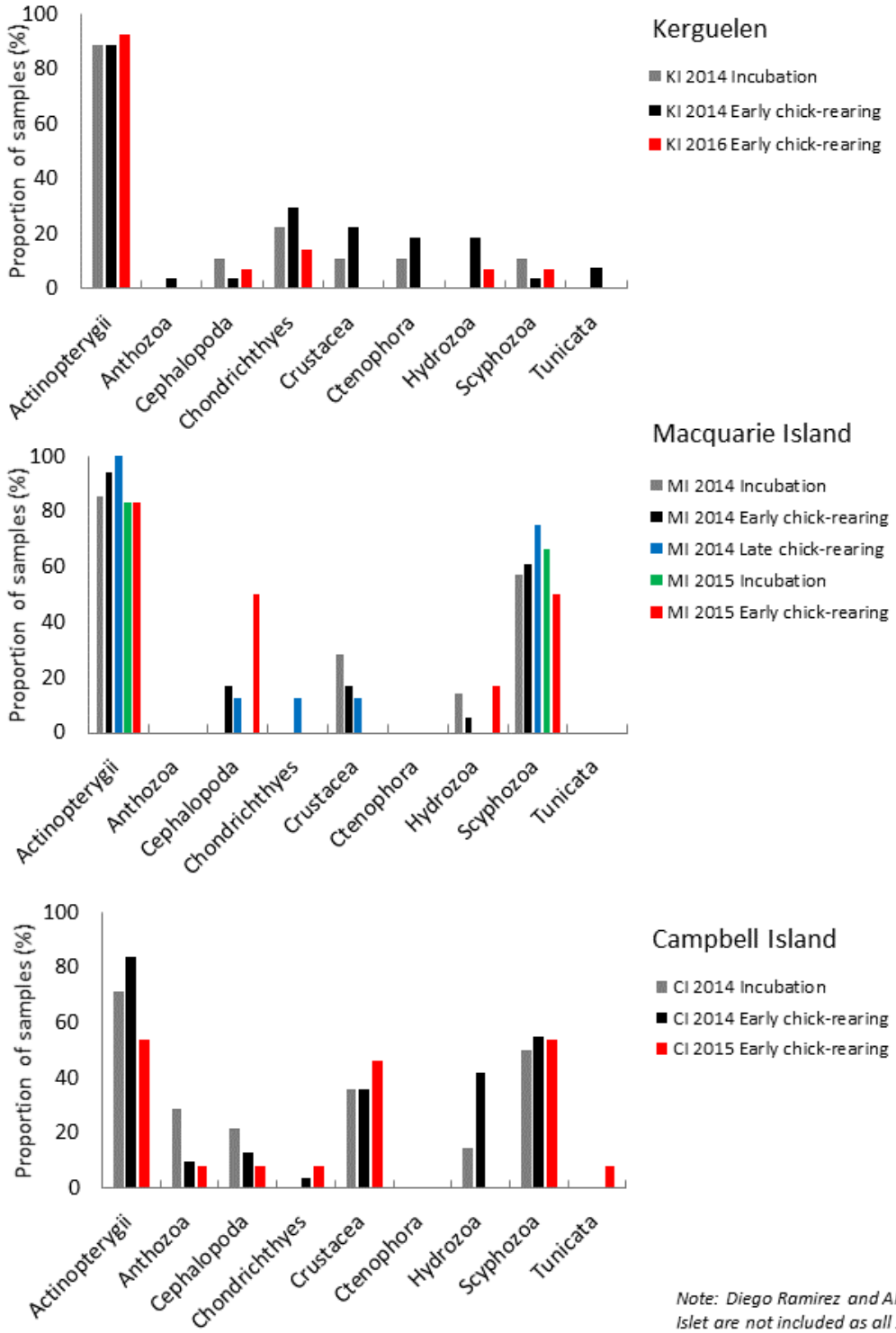
Appendix 4.2: Custom R script for bioinformatics.

```
FastqFolder ="E:/DietData"
setwd (FastqFolder)
dir()
list.files(FastqFolder,pattern=".fastq")

Merge_FQ_Folder = function(FastqFolder,maxee=1.0) {setwd (FastqFolder)
 filenames=list.files(FastqFolder,pattern=".fastq")
 Read1=filenames[grep("R1",filenames)]
 Read2=filenames[grep("R2",filenames)]
 assign("SampNum",length(Read1), env = .GlobalEnv)
 for( i in 1:SampNum)
 {
  system (paste("usearch_v8_0_1623 ",
            " -fastq_mergepairs ", Read1[i],
            " -reverse ", Read2[i],
            " -fastqout"," merged_",formatC(i,width = 3, format = "d", flag = "0"),"_",Read1[i],
            " -fastq_merge_maxee ",maxee,sep=""))
 }}
Merge_FQ_Folder(FastqFolder,maxee=1.0)

library(ShortRead)
FilesMerged=list.files(FastqFolder,pattern=".fastq")[grep("merged",list.files(FastqFolder,pattern=".fa
stq"))]
FilesMerged

All_seq<-list()   # an empty list
for( i in 1:n){
 SeqList=FilesMerged[i]
 All_seq[[i]] = readFastq(FilesMerged[i])  #
}
All_seq

For_Primer="GGTCTGTGATGCCCTTAGATG"
Rev_Primer= "GGTGTGTACAAAGGGCAGGG"
Rev_Primer_RC=reverseComplement(DNAString(Rev_Primer))
DegenerateFor=0
DegenerateRev=0

Tag_seq<-list()
Tag_seq_Trim<-list()

for( i in 1:length(All_seq)){
 Tag_seq[i]=sread(All_seq[[i]])  #Assign which sequence reads to look at
 Tag_seq_Trim[i]= DNAStringSet(Tag_seq[[i]], start=1, end=width(Tag_seq[[i]]))
 print(i)
}

for( i in 1:length(Tag_seq)){
 reads=Tag_seq_Trim[[i]]
```

```
  matchF_List=vwhichPDict
(DNAStringSet(For_Primer),DNAStringSet(substr(reads,1,nchar(For_Primer))), max.mismatch=0,
fixed=FALSE)
  matchF_Index = as.vector(1:length(reads))[unlist(lapply(matchF_List, is.null))==0]
  print(paste("Seq File:",i,"Total Sequences =",length(reads)), quote = FALSE)
  print(paste(round(100*(length(matchF_Index)/length(reads)),2),"% match Forward Primer"), quote
= FALSE)
  matchR_List=vwhichPDict (DNAStringSet(Rev_Primer_RC),DNAStringSet(substr(reads,(width(reads)-
(nchar(Rev_Primer_RC)-1)),width(reads))), max.mismatch=0, fixed=FALSE)
  matchR_Index = as.vector(1:length(reads))[unlist(lapply(matchR_List, is.null))==0]
  print(paste(round(100*(length(matchR_Index)/length(reads)),2),"% match Reverse Primer"), quote
= FALSE)
  match_All_Index=intersect(matchF_Index,matchR_Index)
  print(paste(round(100*(length(match_All_Index)/length(reads)),2),"% match Both Primers"), quote
= FALSE)
  print(paste("Seqs with primers =",length(match_All_Index)), quote = FALSE)
  print("---------------------------------", quote = FALSE)
  match_reads=reads[match_All_Index]
  match_reads_trim=trimLRPatterns(Lpattern = For_Primer, Rpattern=Rev_Primer_RC,
subject=match_reads, max.Lmismatch=DegenerateFor, max.Rmismatch=DegenerateRev)
  names(match_reads_trim)=paste("Seq",1:length(match_reads_trim),sep="_")
  writeXStringSet(match_reads_trim, file=paste("Sample",i,".fasta",sep=""), format="fasta")
  writeXStringSet(match_reads_trim, file="All_processed_seq.fasta", format="fasta", append=TRUE)
}

system ("usearch_v8_0_1623.exe -derep_fulllength All_processed_seq.fasta -fastaout
All_processed_seq_DeRep.fasta -sizeout -minuniquesize 2")
system ("usearch_v8_0_1623.exe -cluster_otus All_processed_seq_DeRep.fasta -otus All_otus.fasta
-uparseout All_uniques.up -relabel OTU_ -sizein -sizeout")

for( i in 1:n){
  system (paste("usearch_v8_0_1623.exe -usearch_global Sample",i,".fasta -db All_otus.fasta -strand
plus -id .97 -uc Sample",i,".uc",sep=""))
}
RefSeqs=readDNAStringSet("All_otus.fasta", format="fasta")
RefTaxa= c("*",names(RefSeqs))
RefTaxa
DataSummary=data.frame(RefTaxa=RefTaxa)
for( i in 1:n){
  UC_file = read.table(paste("Sample",i,".uc",sep=""),sep = "\t",stringsAsFactors=FALSE)
  colnames(UC_file) =c("Hit","Cluster","V3","Per_ID",5:8,"QuerySeq","ClusterLab")
  print(table(UC_file$ClusterLab))
  DataSummary[,(i+1)]= rep(0,length (c("*",names(RefSeqs))))
  Sample1Index=match(names(table(UC_file$ClusterLab)), DataSummary$RefTaxa)
  DataSummary[,(i+1)][Sample1Index]=  table(UC_file$ClusterLab)      #()
}
colnames(DataSummary)=c("RefTaxa",paste("Sample",1:n))
write.csv(DataSummary, file="data.csv")
```

Appendix 4.3


New Island
NI 2014 Early chick-rearing
NI 2015 Incubation
NI 2015 Early chick-rearing
NI 2015 Late chick-rearing
Proportion of samples (%)
0
20
40
60
80
100
Actinopterygii
Anthozoa
Cephalopoda
Chondrichthyes
Crustacea
Ctenophora
Hydrozoa
Scyphozoa
Tunicata
Steeple Jason
SJI 2014 Early chick-rearing
SJI 2014 Late chick-rearing
SJI 2015 Incubation
SJI 2015 Early chick-rearing
SJI 2015 Late chick-rearing
Proportion of samples (%)
0
20
40
60
80
100
Actinopterygii
Anthozoa
Cephalopoda
Chondrichthyes
Crustacea
Ctenophora
Hydrozoa
Scyphozoa
Tunicata
Bird Island
BI 2014 Early chick-rearing
BI 2014 Late chick-rearing
BI 2015 Early chick-rearing
BI 2015 Late chick-rearing
Proportion of samples (%)
0
20
40
60
80
100
Actinopterygii
Anthozoa
Cephalopoda
Chondrichthyes
Crustacea
Ctenophora
Hydrozoa
Scyphozoa
Tunicata



Note: Diego Ramirez and Albatross Islet are not included as all samples were collected during one stage (chick-rearing).

Appendix 4.4 Generalised linear model results for dietary comparisons between site and year overall, and year and breeding stage comparisons at each site. Bolded values are the best model fit. Diego Ramirez and Albatross Islet are not listed in the within site comparisons as only one year of data were collected during one breeding stage.

All sites

				Chi-square	
	df	AIC	df	Dev	p
Base model	22	636.6035			
Year	38	615.573	16	53.03	<0.001
Site	**78**	**490.5963**	**56**	**258.01**	**<0.001**
Year:Site	94	503.7471	16	18.849	0.2766

Bird Island

				Chi-square	
	df	AIC	df	Dev	p
Base model	**12**	**100.5**			
Year	20	106.5	8	10.02	0.26
Stage	20	112.3	8	4.21	0.83
Year:Stage	28	117.7	8	10.59	0.23

Macquarie Island

				Chi-square	
	df	AIC	df	Dev	p
Base model	**13**	**103.7393**			
Year	21	114.5564	8	5.1829	0.7379
Stage	29	125.958	16	9.7812	0.8778
Year:Stage	37	136.221	8	5.7371	0.6767

Kerguelen

				Chi-square	
	df	AIC	df	Dev	p
Base model	**11**	**93.57404**			
Year	19	99.76774	8	9.8063	0.2789
Stage	19	105.2381	8	4.3359	0.8256
Year:Stage	27	109.7745	8	11.464	0.1768

New Island

				Chi-square	
	df	AIC	df	Dev	p
Base model	**12**	**81.75986**			
Year	20	91.82562	8	5.9342	0.6546
Stage	28	108.4401	16	5.3197	0.9939
Year:Stage	36	118.1478	8	6.2924	0.6145

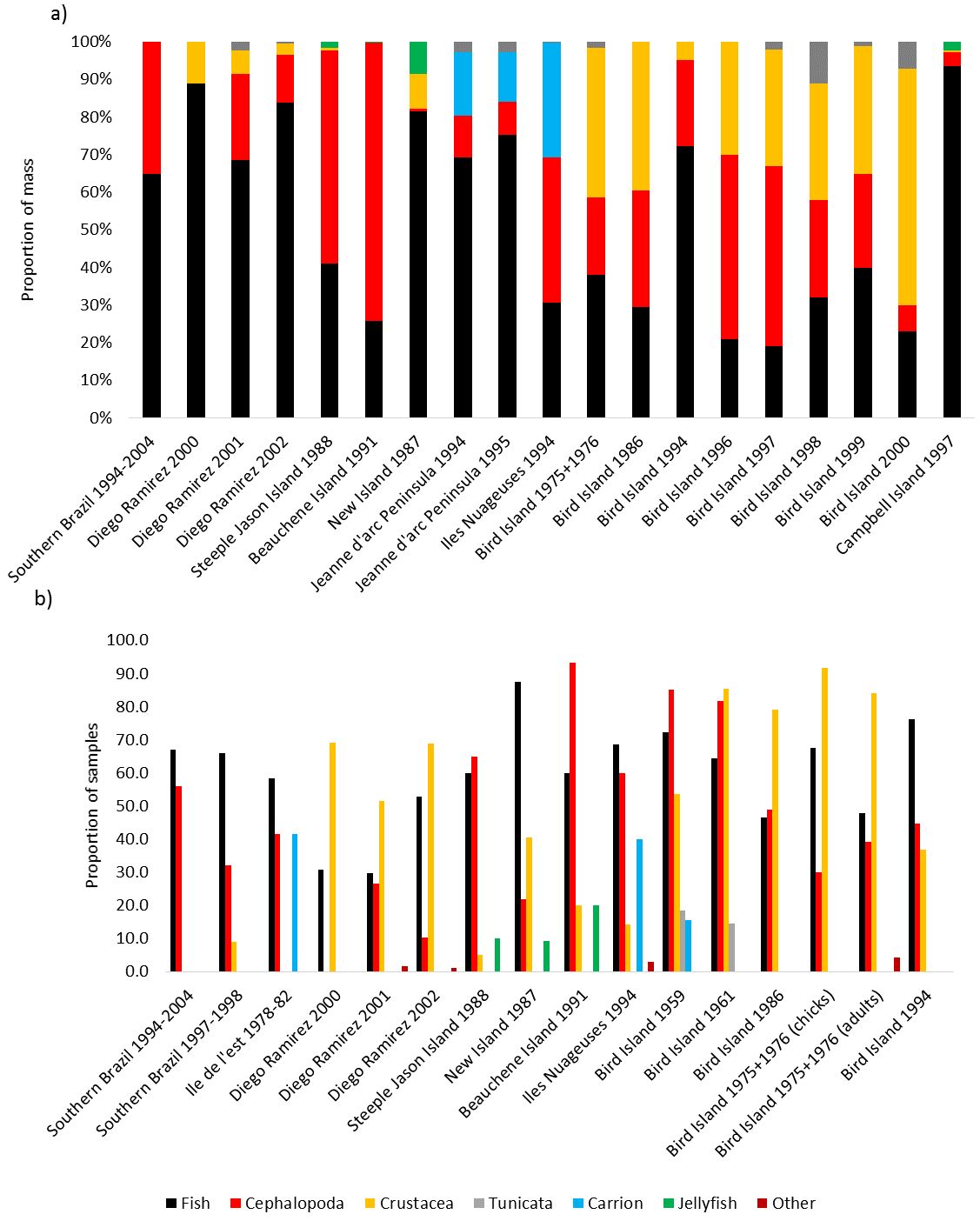
Steeple Jason

				Chi-square	
	df	AIC	df	Dev	p
Base model	**13**	**143.3495**			
Year	21	150.5562	8	8.7933	0.36
Stage	29	152.0969	16	23.253	0.1071
Year:Stage	37	161.664	8	6.4328	0.5989

Campbell Island

				Chi-square	
	df	AIC	df	Dev	p
Base model	**11**	**106.5508**			
Year	19	110.1236	8	12.427	0.1331
Stage	19	116.5305	8	6.0203	0.645
Year:Stage	27	120.2966	8	12.234	0.1411

Appendix 4.5



References: (Tickell 1964, Prince 1980, Weimerskirch et al. 1986, Thompson 1992, Croxall et al. 1997, Waugh 1998, Croxall et al. 1999, Cherel et al. 2000b, 2002, Arata and Xavier 2003, Xavier et al. 2003a, Colabuono and Vooren 2007, Petry et al. 2007)

Chapter 5 - DNA metabarcoding as a marine conservation and management tool: a circumpolar examination of fishery discards in the diet of threatened albatrosses

Submitted as:

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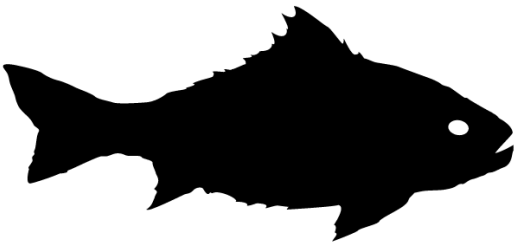

"And a good south wind sprung up behind; the albatross did follow,
And every day, for food or play, came to the mariners' hollo!"

Samuel Taylor Coleridge
The Rime of the Ancient Mariner

5.1 Abstract

Almost all of the world's fisheries overlap spatially and temporally with foraging seabirds, with impacts that range from food supplementation (through scavenging behind vessels), to resource competition and incidental mortality. The nature and extent of interactions between seabirds and fisheries vary, as does the level and efficacy of management and mitigation. Seabird dietary studies provide information on prey diversity and often identify species that are also caught in fisheries, providing evidence of linkages which can be used to improve ecosystem based management of fisheries. However, species identification of fish can be difficult with conventional dietary techniques. The black-browed albatross (*Thalassarche melanophris*) has a circumpolar distribution and has suffered major population declines due primarily to incidental mortality in fisheries. We use DNA metabarcoding of black-browed albatross scats to investigate their fish prey during the breeding season at six sites across their range, over two seasons. We identify the spatial and temporal diversity of fish in their diets and overlaps with fisheries operating in adjacent waters. Across all sites, 51 fish species from 33 families were identified, with 23 species contributing >10% of the proportion of samples or sequences at any site. There was extensive geographic variation but little inter-annual variability in fish species consumed. Several fish species that are not easily accessible to albatross, but are commercially harvested or by-caught, were detected in the albatross diet during the breeding season. This was particularly evident at the Falkland Islands and Iles Kerguelen where higher fishery catch amounts (or discard amounts where known) corresponded to higher occurrence of these species in diet samples. This study indicates ongoing interactions with fisheries through consumption of fishery discards, increasing the risk of seabird mortality. Breeding success was higher at sites where fisheries discards were detected in the diet, highlighting the need to minimise discarding to reduce impacts on the ecosystem. DNA metabarcoding provides a valuable non-invasive tool for assessing the fish prey of seabirds across broad geographic ranges. This provides an avenue for fishery resource managers to assess compliance of fisheries with discard policies and the level of interaction with scavenging seabirds.

5.2 Introduction

Effective ecosystem-based management of commercial fisheries requires information not just on the sustainability of target stocks, but also on the interactions of other marine organisms with fishing operations. Globally, seabirds frequently interact with commercial fisheries through competition for shared resources (Frederiksen et al. 2004, Okes et al. 2009), incidental mortality in fishing gear (Brothers et al. 1999a, Sullivan et al. 2006, Watkins et al. 2008, Tuck et al. 2011) and consumption of fishery discards (Garthe et al. 1996, Gonzalez-Zevallos and Yorio 2006). Seabird survival and breeding success can be reduced by competition with fisheries (Furness and Tasker 2000, Frederiksen et al. 2004), and incidental mortality in fishing gear can be a major cause of population declines, particularly of albatrosses and large petrels (Weimerskirch and Jouventin 1987, Barbraud et al. 2008, Phillips et al. 2016). Physical and operational mitigation measures have been developed to reduce seabird mortality (Løkkeborg 2008, Phillips et al. 2016), including the reduction of fishery discards, which decreases the attractiveness of vessels (Abraham et al. 2009, Pierre et al. 2012). Scavenging birds are attracted to the supplementary food source provided by discards, which may consist of (i) the head, tail and offal of retained catch (commercial species caught at commercial size); (ii) whole fish of commercial species but caught at a non-commercial size; (iii) non-commercial species and (iv) unused baits (in longline fishing). These discards are often fish or other species that may not be naturally accessible. Some populations benefit from the additional food source, with higher breeding success and survival resulting in population growth (Oro et al. 1995, Bertellotti and Yorio 2000). However, discards can alter food-web structure by providing nutritionally-poor food (Grémillet et al. 2008), or artificially inflating populations of predatory gulls or skuas, which may not be sustainable in the absence of discards or which also prey on smaller seabirds, with potentially major impacts (Phillips et al. 1999b, Foster et al. 2017). The interactions between seabird populations and fisheries are likely to vary over time, space and species; therefore, understanding the nature and extent of these interactions is imperative for effective ecosystem management.

Seabird dietary studies can inform ecosystem risk assessments for fishery management by identifying interactions between fisheries and seabirds for different populations (Phillips et al. 1999a). Understanding the dietary flexibility of seabirds is also fundamental for predicting the responses of individuals and populations to spatial and temporal changes in natural prey abundance, and availability from fisheries, and hence for the effective management of marine resources (Constable et al. 2000). Stomach content and stable isotope analyses are the two main approaches for assessing seabird diet (Duffy and Jackson 1986, Barrett et al. 2007). The former primarily relies on the use of otoliths and bones to identify fish prey, enabling prey size and meal mass estimates to

be obtained. However, discrimination can be poor or impossible if the prey (including larvae or eggs) is small, has no hard parts, or digests quickly; the hard-parts are eroded; or those from closely-related species cannot be readily distinguished (Duffy and Jackson 1986, Barrett et al. 2007). These problems apply in particular to items originating as fisheries offal, as viscera float and are therefore easier to ingest than fish heads with otoliths, particularly those from large species (Thompson and Riddy 1995). More recent studies have used DNA analysis to identify parts that were not taxonomically diagnostic (Alonso et al. 2014). However, studies using stomach samples are usually restricted to the chick-rearing period, thus focusing on chick rather than adult diet across the annual cycle and usually requires handling of birds.

Stable isotope analysis of blood or feathers does not suffer from the biases associated with differential digestion of prey and can be applied to all stages of the breeding season. This method has been used to determine likely fishery overlaps by comparing the estimated proportions of pelagic and demersal prey, on the assumption that the latter were obtained from fisheries (Granadeiro et al. 2013). However, in most systems stable isotope analyses lack the resolution to identify prey beyond broad trophic groups. DNA metabarcoding of predator scats is a useful alternate or complementary method for assessing seabird diet (Deagle et al. 2007, Bowser et al. 2013). It can provide high-level taxonomic resolution and does not require prey remains to be physically identifiable (Pompanon et al. 2012). Although the method cannot be used to identify prey size and meal mass, it does give an indication of species occurrence in the diet. Samples can also be collected during all breeding stages (McInnes et al. 2017a) and the technique is non-invasive and requires minimal field time compared to conventional diet sampling, increasing the options for simultaneous sampling across broad spatial scales (Jarman et al. 2013).

The black-browed albatross (BBA, *Thalassarche melanophris*) has a circumpolar distribution and is the most abundant albatross species in the southern hemisphere (Phillips et al. 2016). Populations have experienced extensive declines which are strongly linked to incidental mortality in longline and trawl fisheries (Phillips et al. 2016). While the population at South Georgia is still declining (Poncet et al. 2017), numbers in the Falkland Islands and on islands off Chile are currently increasing (Wolfaardt 2013, Robertson et al. 2014, Robertson et al. 2017). The increases in Chile have been attributed to a reduction in incidental seabird mortality due to faster sink rates of baited longline hooks associated with a change in fishing practices, and the use of bird-scaring (streamer or tori) lines, making hooks less accessible to birds (Robertson et al. 2014). However, longline and trawl fisheries are still thought to cause high mortality of this species elsewhere, especially in the wintering grounds (Yeh et al.

2013, Kuepfer 2015, Tamini et al. 2015). Fishery resource overlaps with the diet of black-browed albatrosses have been shown at all breeding sites where fish have been characterised, including Iles Kerguelen (Cherel et al. 2000b), Diego Ramirez (Arata and Xavier 2003), South Georgia (Reid et al. 1996, Xavier et al. 2003a) and the Falkland Islands (Thompson 1992). However, the most recent samples used in these studies were collected over 15 years ago (1995, 2002, 2000 and 1991 respectively; Appendix 5.1), over which time fishing operations and regulations, including discarding policies and mitigation requirements, have changed substantially in many regions (Phillips et al. 2016).

We used DNA metabarcoding of BBA faecal DNA to investigate the fish prey consumed at six sites across their breeding range to: 1) determine the fish prey diversity and any spatial and temporal variability; 2) identify any fishery target, bycatch and bait species in the diet of BBA to distinguish regions in which rates and risks of vessel interactions may be greater (and hence efforts to improve discard management and monitoring of compliance with seabird bycatch mitigation may be targeted); and 3) evaluate sources of potential resource competition or food supplementation by fisheries. We use this study to show that DNA metabarcoding can quantify fish diversity and the presence of discards in the diet of seabirds, providing a valuable tool for fishery resource and conservation management.

5.3 Methods

5.3.1 Study sites and sample collection

Fresh scat samples were collected from black-browed albatrosses at six breeding colonies over multiple seasons: in austral summers 2013/14 and 2014/15 at New Island and Steeple Jason Island (Falkland Islands), Macquarie Island (Australia) and Bird Island (South Georgia); in 2013/14 and 2015/16 at Canyon des Sourcils Noirs (Iles Kerguelen); and in 2014/15 at Albatross Islet (Chile; Figure 5.1). The majority of samples were collected during the chick-rearing period (December-March) with additional samples collected during incubation in 2014/15 at Steeple Jason Island and New Island, Kerguelen in 2013/14 and during incubation in both years at Macquarie Island (Table 5.1). Sampling years are hereafter termed 2014 for samples collected in 2013/14 and 2015 for 2014/15 samples. This project was approved by the University of Tasmania Animal Ethics Committee (Permit A13745).

A small fragment of the non-uric acid portion (dark part) of each scat was collected using tweezers or a spatula and stored in 80% ethanol. Where possible, fresh scats were obtained (where the bird

was seen defecating or the sample was on the ground but still wet) and the developmental stage of the bird (chick, juvenile or adult) was recorded. Given the low sample sizes remaining when samples were split by site, age and month, differences between diet of chicks and adults (self-feeding) could not be explored in this study, and therefore samples from different ages were pooled. Further research with greater sample sizes are required to test partitioning of diet by adults for provisioning compared with self-feeding (Davoren and Burger 1999, Danhardt et al. 2011), and potential dietary differences between breeders, non-breeders and juveniles (Campioni et al. 2016).

The foraging ranges of black-browed albatross are greater during incubation than chick-rearing, and the magnitudes of these differences depend on the colony (Wakefield et al. 2011). For example, at South Georgia, mean maximum foraging distances of tracked adults were 980-1690km (262-327 hours) and 275-505km (45-77h) during incubation and chick-rearing, respectively (Phillips et al. 2004). The prey detected in scat samples is likely to reflect the most recent meal consumed by albatross, which is similar to stomach contents analysis. The digestion rates of seabirds are influenced by numerous variables, such as predator species, metabolic rate, meal size, food type and feeding frequency (Hilton, Houston & Furness 1998). In sooty albatross (*Phoebetria fusca*), the mean retention rate of prey ranged from 11-15 hours, however some prey was still detected up to 50 hours after eating (Jackson 1992). In little penguins (*Eudyptula minor*) prey could be detected for up to four days using DNA metabarcoding (Deagle et al. 2010). The retention time is also likely to vary depending on whether the food is consumed for self-feeding or regurgitated to the chick partially digested. During this study, it is assumed that the prey DNA recovered reflects the most recent foraging trip. For extended foraging trips during incubation, some of the food may not be detected.

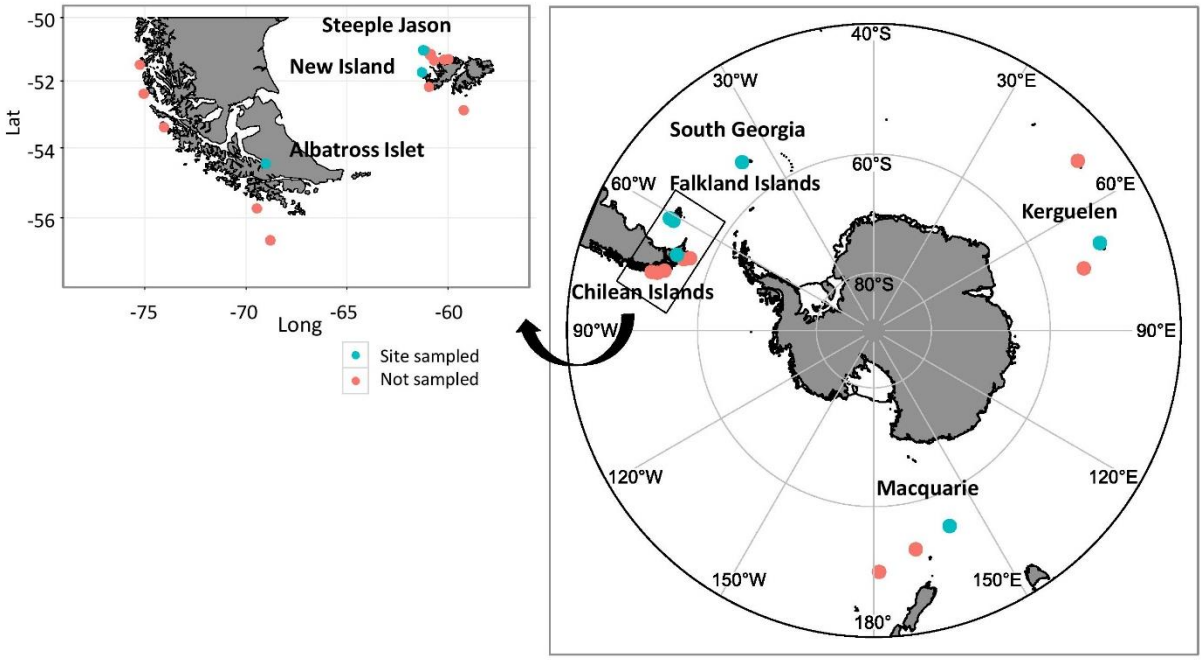

Figure 5.1: Breeding distribution of black-browed albatrosses and sampling sites. Blue dots represent the six colonies where scat samples were collected, and the red dots the remaining colonies not sampled during the study. The inset shows the individual Chilean and Falkland Island colonies. Samples were collected from Albatross Islet, Chile (40-50 breeding pairs, population increasing); New Island (13,343 breeding pairs, population increasing) and Steeple Jason Island (183,135 pairs, population increasing), Falkland Islands; Bird Island, South Georgia (8,264 breeding pairs, declining); Canyon des Sourcils Noirs, Iles Kerguelen (~1000 breeding pairs, population stable); and Macquarie Island (~200 breeding pairs, population stable; ACAP 2010, Wolfaardt 2013, Robertson et al. 2014, Phillips et al. 2016, Poncet et al. 2017).

Table 5.1 The total samples at each site which contained food DNA derived from the 18S_SSU primer set and 16S_Fish primer set. Values in brackets represent the number of samples from chicks. The frequency of occurrence (FOO, %) and relative read abundance (RRA, %) of fish are presented for those samples from which greater than 100 food sequences amplified with the 18S_SSU primer set. Fish includes Actinopterygii (bony fish) and Chondrichthyes (sharks and skates). Sample sizes presented for each breeding stage: incubation (INC), early chick-rearing (ECR) and late chick-rearing (LCR).

Site	Year	FOO of fish sequences (18S) *	RRA of fish sequences (18S) *	Number of samples with food DNA (18S)*	Number of samples amplified (16S)#										
					Incubation			Early chick-rearing		Late chick-rearing		**Total**			
					Oct	Nov	Early Dec	Late Dec	Jan	Feb	Mar	**INC**	**ECR**	**LCR**	**All**
Albatross Islet	2014/15	100.0	97.2	51						49				**49**	**49**
New Island	2013/14	95.5	67.9	22				12 (4)	7 (2)				**19(6)**		**19 (6)**
	2014/15	92.9	75.7	28	2	10	6	1	2	5(3)		**18**	**3**	**5 (3)**	**26 (3)**
Steeple Jason Island	2013/14	72.9	40.8	59					15		14 (14)		**15**	**14 (14)**	**29 (14)**
	2014/15	77.5	58.2	40	13				2		7 (7)	**13**	**2**	**7 (7)**	**22 (7)**
Bird Island	2013/14	90.9	76.4	33					17	4 (1)	6 (1)		**17**	**10 (2)**	**27 (2)**
	2014/15	92.9	79.3	42					39		2		**39**	**2**	**41**
Iles Kerguelen	2013/14	100.0	92.4	36		3	6	1	20			**9**	**21**		**30**
	2015/16	100.0	93.4	16					16				**16**		**16**
Macquarie Island	2013/14	93.9	59.4	33		6	3	6	6(5)	2 (1)	3 (3)	**9**	**12 (5)**	**5(4)**	**26 (9)**
	2014/15	83.3	54.9	12		3	2		4(4)		1 (1)	**5**	**4 (4)**	**1(1)**	**10 (5)**
Overall average			**90.9**	**73.3**	**372**	**15**	**22**	**17 (4)**	**20 (4)**	**128 (11)**	**60**	**33**	**54**	**148 (9)**	**93 (31)**

* Samples with >100 food sequences; # Samples with >100 fish sequences

5.3.2 DNA metabarcoding

DNA was extracted from albatross scat samples using a Promega 'Maxwell 16' instrument and a Maxwell® 16 Tissue DNA Purification Kit. PCR inhibitor concentrations were diluted by mixing a small amount (~30mg) of the faecal samples in 250µl of STAR buffer (Roche Diagnostics) prior to extraction. Two different DNA markers were amplified. The first used a metazoan primer set that is highly conserved and amplifies a region of the nuclear small subunit ribosomal DNA (rDNA) 18S gene (McInnes et al. 2017a, Table 2). For this marker the taxonomic resolution is relatively low; however, it recovers DNA from all animal lineages and provides a broad view of the diet. The second primer pair amplifies a region of the 16S rDNA gene specifically from fish and varies enough to allow species-level identification for most of the targeted fish species (Table 5.2). This primer set was designed based on an alignment of mtDNA 16S sequences from representative Southern Ocean fish that were publically available on Genbank. The primer set was designed not to match bird DNA. Primers were tested with fish flesh and scat DNA. All samples were run with the 18S_SSU primer set first, and those that had fish DNA were amplified using the 16S_Fish primer set (Figure 5.2).

PCR reactions for each primer set were carried out separately as a two stage process. Stage one PCR reactions (10 µL) were performed with 5 µL 2 x Phusion HF (NEB), 1 µL 100 x Bovine Serum Albumin (NEB), 0.1 µL 5 µM of each 18S_SSU or 16S_Fish amplification primer (Table 5.2), 0.5 µL of Evagreen, 2 µL faecal DNA and 1.3 µL of water. Thermal cycling conditions were 98^{o}C, for 2 mins; followed by 35 cycles for 18S_SSU, and 45 cycles for 16S_Fish, of 98^{o}C for 5 s, 67^{o}C for 20 s, 72^{o}C for 20s, with an extension of 72^{o}C for 1 min. Each sample was run in triplicate on a LightCycler 480 (Roche Diagnostics). A negative control containing no template DNA and positive control containing fish DNA were included in each PCR amplification run. If either the negative amplified or the positive failed to amplify, the PCR was re-run. If ≥2 replicates of each sample had a ct score < 30 for the 18S_SSU, or < 40 for 16S_Fish, they were combined to reduce biases produced by amplification from samples with low template concentrations (Murray et al. 2015). Pooled samples were diluted 1:10 for the second stage PCR. In the second stage PCR, a unique tag was attached to each sample (Table 5.2). PCR reactions (10 µL) were performed with 5 uL 2 x Phusion HF (NEB), 1 µL 100 x Bovine Serum Albumin (NEB), 1 µL of 1 µM of each tag primer, and 2 µL of diluted PCR product from stage one. Thermal cycling conditions were 98^{o}C, for 2 min; followed by 10 cycles of 98^{o}C for 5 s, 55^{o}C for 20 s, 72^{o}C for 20 s, with an extension of 72^{o}C for 1 min. Samples were pooled and purified from unincorporated reaction components by washing, utilising reversible binding to Ampure (Agencourt) magnetic beads following the manufacturer's protocol. Sequencing of PCR products was performed with an Illumina MiSeq high throughput sequencer, using the MiSeq reagent kit V2 (300 cycles).

Table 5.2 Oligonucleotides used in this study. Underlined bases in PCR Round 1 are the Miseq tag primer. Bolded bases in PCR Round 2 are an example of the unique tags attached to each sample.

PCR Round	Primer Name	Primer sequence (5'-3')	Fragment length	Reference
1	18S_SSU_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTCTGTGATGCCCTTAGATG	~170bp	McInnes et al 2017
1	18S_SSU_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGTGTGTACAAAGGGCAGGG		McInnes et al 2017
1	16S_Fish_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCGYAATCACTTGTCTYTTAA	~200bp	This study
1	16S_Fish_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCRBGGTCGCCCCAACCRAA		This study
2	SSU_Tag_F1	AATGATACGGCGACCACCGAGATCTACAC**A GTTCGGACT**TCGTCGGCAGCGTC	~150bp	Jarman et al 2013
2	SSU_Tag_R1	CAAGCAGAAGACGGCATACGAGAT**AGCTTA GGCT**GTCTCGTGGGCTCGG		Jarman et al 2013

5.3.3 Bioinformatics

Amplicon pools were de-multiplexed based on unique 10 bp Multiplex IDentifiers (MIDs) incorporated in the Illumina two-step MID protocol. Fastq files were processed using USEARCH v8.0.1623 (Edgar 2010). Reads R1 and R2 from the paired end sequencing were merged using the fastq_mergepairs function, retaining only merged reads flanked by exact matches to the primers and primer sequences were trimmed. Reads from all samples were pooled and dereplicated, then clustered into broad Operational Taxonomic Units (OTUs) using the cluster_otus command (-otu_radius_pct = 10). Potentially chimeric reads were discarded during this step. Reads for each sample were assigned to these OTUs (usearch_global -id 0.97) and a summary table generated using a custom R script. Each OTU was identified by BLAST and categorised to closest match using MEGAN 5 (Huson et al. 2007) and the Lowest Common Ancestor (LCA) assignment algorithm. LCA parameters were set at a minimum score of 250 and a top-percent of 5% for the 18S_SSU and 340 and 5% for the 16S_Fish. These cut-offs were determined by manually checking a subset of samples against BLAST. Sequences were also manually checked on Genbank to ensure that all species from that genus in the region were represented. Additional flesh samples were obtained at the Falkland Islands (Gras et al. 2016) and through the Australian Antarctic Division and were sequenced and added to Genbank (see data Availability section for accession numbers) .

OTUs derived from the 18S_SSU primer set were assigned to class, whereas OTUs derived from the 16S_Fish primers were classified to genus or species. OTUs were assigned only to genus if there was any uncertainty in the species match, either due to insufficient difference between species in the

16S region amplified, or if species from that genus were not present on Genbank. The geographic distribution of species in each genus was checked in Gon and Heemstra (1990) and Duhamel et al. (2014), and species was assigned if only one occurred within the foraging range of BBA from a particular site. In such cases, the species name in tables and figures is given in parentheses. Samples amplified with the 18S_SSU primers were included if they contained at least 100 sequences of food DNA, whereas samples amplified for the 16S primers were included if they contained at least 100 sequences of fish DNA (Jarman et al. 2013). Results are presented as the number of samples with a prey item (n), the frequency of occurrence (FOO) and the relative read abundance of sequences (RRA). For FOO calculations, any food item or fish species was deemed present if it comprised >1% of food sequences for 18S_SSU, or fish sequences for 16S_Fish. The RRA for 18S was calculated as the total sequences for that prey group divided by the total food sequences for that sample, whereas the RRA for the 16S was the number of sequences for a fish species divided by the total fish sequences for that sample. The RRA was averaged across island or year groups. These multiple measures of diet composition are presented to reduce potential biases in interpretation that might result from consideration of a single metric. The results from the 18S region are presented to show the fish component of the diet and allow calculations of the overall proportion of the population consuming discards. Further details and discussion on the proportions of each prey group for each site can be found in McInnes et al. (2017b).

5.3.4 Assessing overlaps between commercial fisheries and BBA prey

Data on fishery catches and target species were provided by the Directorate of Natural Resources of the Falkland Islands Government; the Australian Fisheries Management Authority and the Australian Antarctic Division; the Pecheker database (Martin and Pruvost 2007) and online Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) Statistical Bulletins (CCAMLR 2015). These included fishing effort (total hours for trawl and total hooks for longline); the total catch of target species and the main bycatch species (those comprising > 1% of the total catch); the fish bait used in long-line fishing operations; and, at Iles Kerguelen, the mass of target and bycatch species that were discarded. No data were available on Illegal, Unreported and Unregulated (IUU) fishing. During the relevant sampling periods, trawl and longline fisheries were operating during the sampling period within the Falkland Islands Inner and Outer Conservation Zones (FICZ/FOCZ; Table 5.3), with no trawl fishing during January; longline but no trawl fisheries were operating within the Kerguelen Economic Exclusion Zone (EEZ); trawl but no longline fisheries operated close to South Georgia during the sampling period (CCAMLR Division 48.3; excluding March); no fishery was operating in the Macquarie Island EEZ; nor was there a fishery within the Admiralty Sound or

Magellan Strait, which are used by foraging birds from Albatross Islet during chick rearing (Arata et al. 2014). Fishery species were defined as any target fish species, or bycatch fish species that made up greater than 1% of the total catch (Table 5.3). Bait species used during fishing operations were also identified. For the main fish species (those contributing > 10% of amplified sequences), the depth profile for each species during different age stages were compiled from the literature to determine which were likely to be naturally accessible to albatrosses (Appendix 5.2). This study focused on the fishing zones adjacent to the breeding sites, as these are likely to be used more intensively than distant waters by foraging birds during chick-rearing (Phillips et al. 2004, Terauds et al. 2006a, Catry et al. 2013, Arata et al. 2014), and secondly, the management of these areas is within the same national jurisdiction as the relevant breeding site. However, we acknowledge that birds may have also interacted with fisheries further from colonies, especially during incubation when BBA are foraging farther from the colony than during chick rearing (Phillips et al. 2004, Wakefield et al. 2011).

5.3.5 Statistical Analyses

Analyses were carried out using R software (R Core Team 2015). Poisson generalised linear models (GLM) with a log link function were used to test if there were differences in fish species composition between colonies and years, and between years and breeding stages at each site. The model included the count of samples (n) as the dependant variable and predictor variables included fish species (F), year (Y) and breeding stage (S), or colony (C). The base model included the sample size as a function of the main effects (fish species, year, breeding stage or colony) as well as the year:stage or year:colony interaction. These terms effectively describe the patterns in the data arising from the experimental sampling process (e.g. total number of samples within a given year). The interaction terms, fish:year, fish:stage or fish:colony were added to the base model to test the effect of year or stage (or colony for the pooled data) on diet composition. The analysis of deviance (with Chi-squared test) and Akaike's information criterion (AIC) were used to compare fitted models and test the significance of predictor terms (Burnham and Anderson 2002). A linear regression was used to assess the relationship between the proportion of samples with discards and breeding success, based on monitoring of BBA nesting attempts at each colony in the year that the diet samples were collected. Dissimilarity indices were calculated with the Manhattan method using the command 'vegdist' in the package 'Vegan' (Oksanen et al. 2016). From these indices, a hierarchical clustering was then constructed using the average agglomeration method, and plots created using the package 'ggplot2' (Wickham 2009). The proportion of samples that amplified with the 16s_Fish primer which

contained species that are also caught in fisheries was calculated, and applied to the total number of samples collected that amplified fish with the 18s_SSU primers.

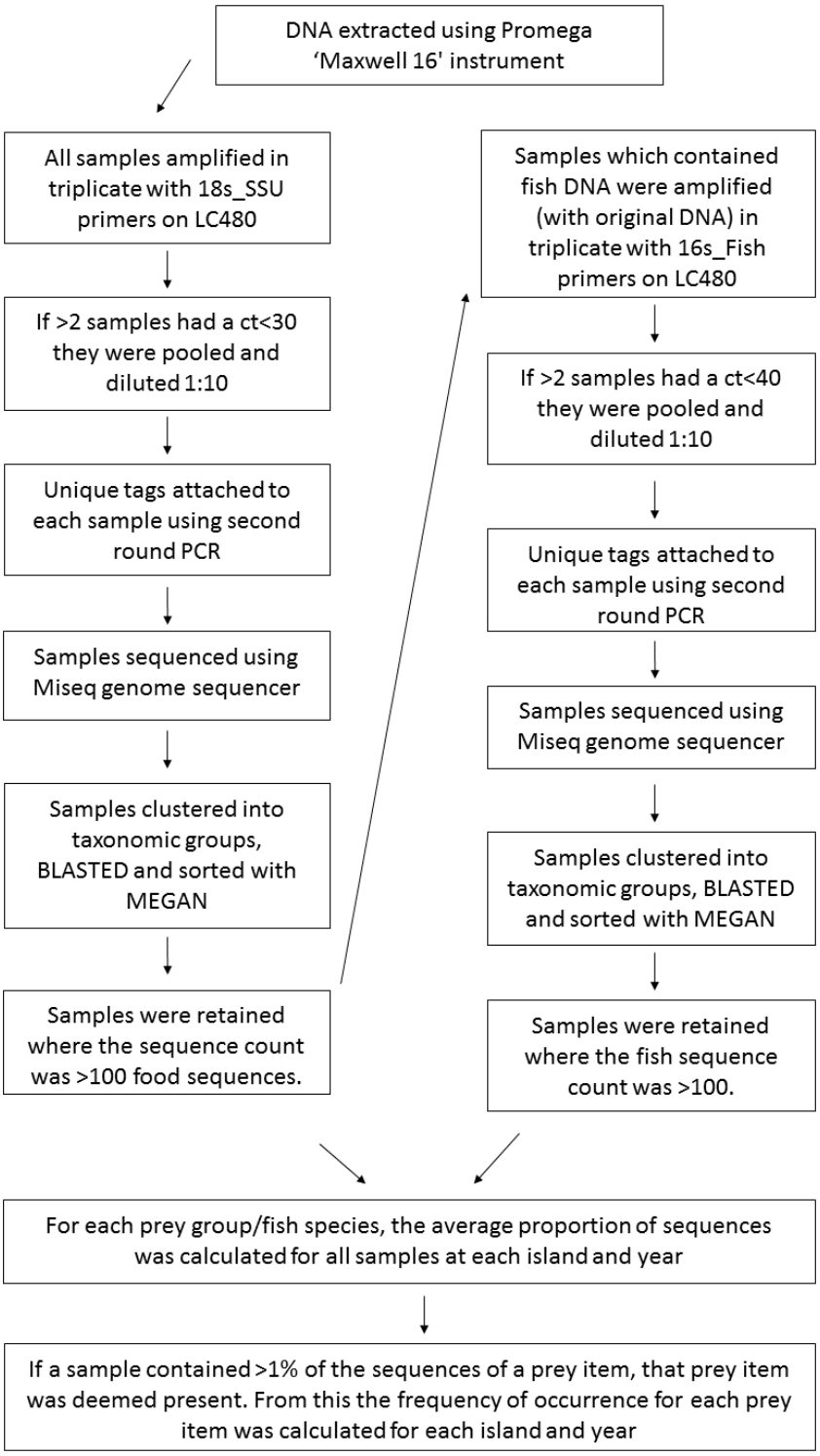

Figure 5.2 Work flow for DNA metbarcoding of BBA scats

Table 5.3 Details of commercial fisheries operating in waters adjacent to breeding colonies of black-browed albatrosses during the sampling periods. The fish species listed are those targeted by the fishery; bycatch species are those that constitute >1% of the total catch.

Island site	Fishery Area	Fishery	Operation dates	Target fish	Main bycatch species	Discard policy	Bait species
Albatross Islet	Admiralty Sound and Magellan strait	Artisanal fisheries	All year	Assorted fish	-	-	-
	FICZ/FOCZ	Long-line	All year	*Dissostichus eleginoides*	Rajiformes	Discards permitted	*Sardina pilchardus* (Squid sp.)
	FICZ/FOCZ	Trawl	All year	*Patagonotothen ramsayi* *Dissostichus eleginoides* *Genypterus blacodes* *Macruronus magellanicus* *Micromesistius australis* *Merluccius* sp. *Salilota australis* Rajidae	Macrouridae	Discards permitted	-
Bird Island	CCAMLR Division 48.3	Pelagic trawl	All Year	*Champsocephalus gunnari*	*Pseudochaenichthys georgianus* *Patagonotothen guntheri* *Notothenia rossii* *Lepidonotothen squamifrons*	Discards prohibited during shooting and hauling (CM 25-03; CCAMLR 2014b)	-
Iles Kerguelen	EEZ	Long-line	March-January	*Dissostichus eleginoides*	Macrouridae Rajiformes *Antimora rostrata*	Discards prohibited during setting (CM 25-02; CCAMLR 2014b)	*Sardinops sagax* *Cololabis saira* *Trachurus trachurus* *Scomber japonicus* *Scomber scombrus* *(Illex argentinus)*

5.4 Results

5.4.1 Diversity, and spatial and temporal variability in fish prey of BBA

A total of 1091 scat samples were collected. DNA was amplified in 793 samples using the 18S_SSU primers; 372 samples contained at least 100 sequences of food DNA, and 327 contained fish DNA. These samples were then amplified with the 16S_Fish group specific primers; 295 samples contained at least 100 fish sequences and were included in subsequent analyses (Table 5.1).

Fish were found to be the most common prey group across all sites and years, based on the 18S_SSU data. In total, 91% of samples contained fish and this made up 72% of sequences (ranging from 73 to 100% of samples, and between 41 - 97% of sequences at different sites; Table 5.1). Chondrichthyes (sharks and skates) were present in 2.7% of samples and comprised 2% of these sequences (Figure 5.3).

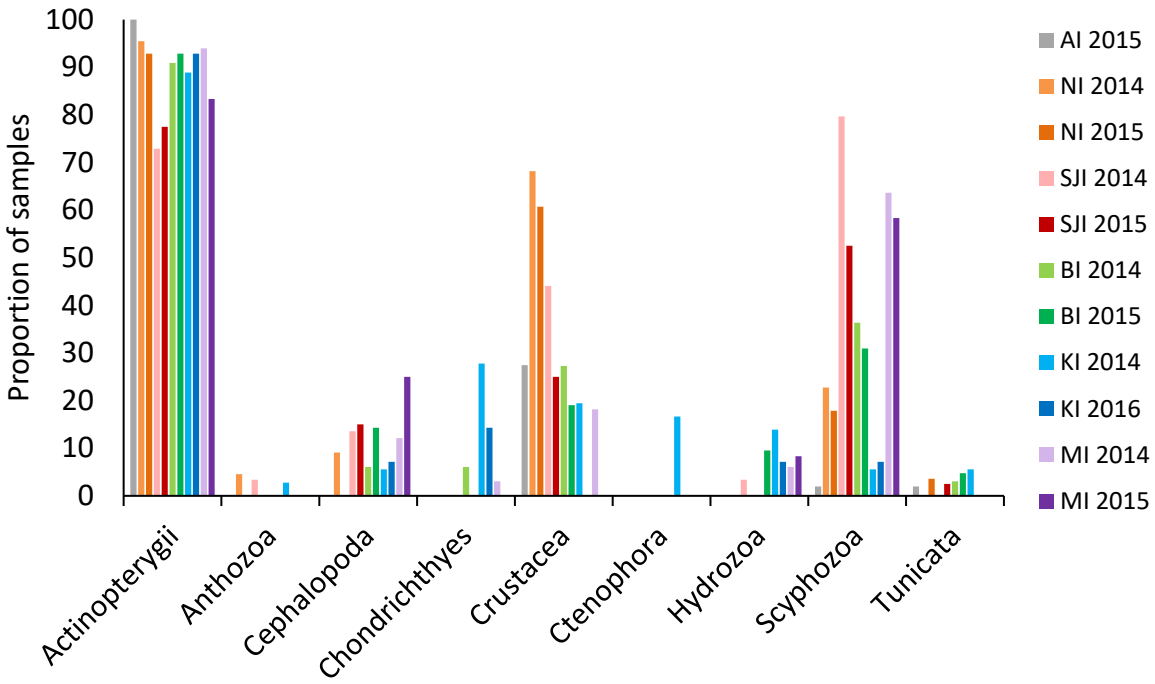

Figure 5.3 Overall diet of black-browed albatross from 2014-2016 using 18S_SSU primers. Sites were New Island (NI) and Steeple Jason Island (SJI), Falkland Islands; Bird Island, South Georgia (BI); Canyon des Sourcils Noirs, Iles Kerguelen (KI); and Macquarie Island (MI). Values represent the frequency of occurrence for each site. Lighter coloured bars correspond to 2014, darker bars to 2015 (or 2016 for Iles Kerguelen). A prey group was considered to be present if it contributed > 1% of the total sequences for that sample.

The higher resolution provided by the mtDNA 16S marker identified at least 51 fish species, from 33 families in the diet of BBA across the six breeding sites, with 23 species constituting >10% of the amplified sequences for different colonies and years (Table 5.4). The most common fish prey belonged to four families: Notothenіidae (notothens), Channichthyidae (crocodile icefishes), Congiopodidae (horsefishes) and Clupeidae (herrings, sardines and allies; Tables 5.5 and 5.6). Colonies were clustered into four distinct groups according to fish species composition: 1) Falkland Islands and Albatross Islet, 2) Iles Kerguelen, 3) Macquarie Island and 4) Bird Island (Figure 5.4). When grouped by family, clusters were similar to fish species, except samples from Steeple Jason in 2015 were more similar to those from Iles Kerguelen due to the high occurrence of Nototheniidae. Fish from the family Nototheniidae were common to all groups. Clupeidae was common in group 1, Channichthyidae in groups 2 and 4, and Congiopodidae in group 3. The differences between years were less marked than those among colonies, as the inclusion of colony provided the best model fit (Base model AIC=1618, F:Y AIC=1560, F:C AIC=1054, F:C+F:Y AIC=1175). Two to eight fish species contributed >10% of the fish prey for each colony-year combination (in either FOO or RRA), and were found in more than one sample (Table 5.4).

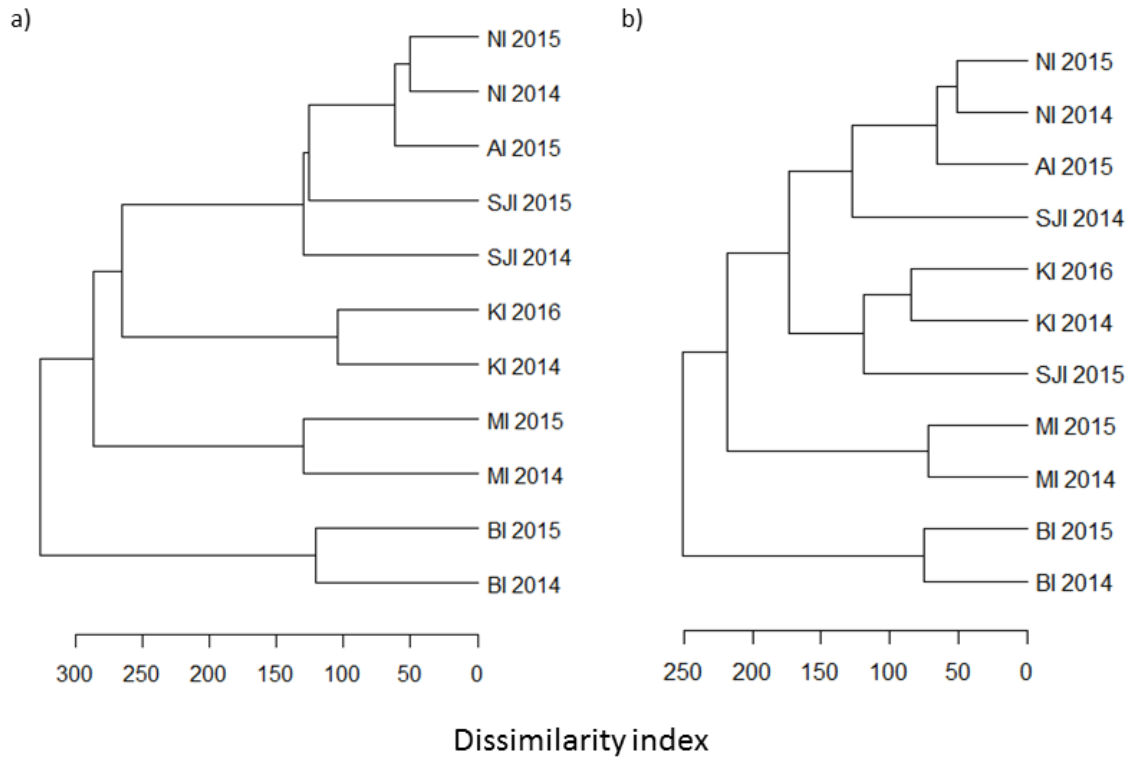

Figure 5.4 Hierarchical clustering of the frequency of occurrence of fish at each site at the level of A) species, and B) family. Clusters were based on dissimilarity indices calculated with the Manhattan method, and hierarchical clustering was constructed using the average agglomeration method.

Albatross Islet

Eight fish species were found in the 49 samples from Albatross Islet; the majority contained Fueguian sprat (*Sprattus fuegensis*; 88% FOO), and black southern cod or Patagonian rockcod (*Patagonotothen tessellata* or *brevicauda*) was the second most common item (29% FOO; Table 5.5, Figure 5.5).

Falkland Islands

Eight fish species were identified in the 45 samples from New Island, almost entirely comprised (>90% of sequences) of Fueguian sprat (68% FOO) and rockcod (*Patagonotothen* sp.; 53% FOO; Table 5.5). There was no difference in the FOO of fish species consumed between years (Base model AIC=80.27, F:Y AIC =84.05; $\chi^2_7 = 10.22$, p = 0.17) or breeding stages (F:S AIC=97.45; $\chi^2_{14} = 10.82$, p=0.70; Figure 5.5).

Ten fish species were identified in the 51 samples from Steeple Jason Island, of which sprat was the most common species in 2014 (48% FOO), followed by hoki (*Macruronus magellanicus;* 21% FOO), southern blue whiting (*Micromesistius australis australis*; 21% FOO), rockcod (17% FOO) and kingclip (*Genypterus blacodes;* 10% FOO; Table 5.5). In 2015, rockcod was the main item (64% FOO) followed by sprat (18% FOO) and hoki (14% FOO). There was a difference in the fish species consumed between years (Base model AIC=157.4, F:Y AIC=152.51; $\chi^2_9 = 22.90$, $p < 0.01$) and breeding stages (F:S AIC =129.3; $\chi^2_{18} = 64.13$, $p<0.001$). When data were adjusted for year, the effect of stage was still significant (F:Y and F:S AIC =142.5; $\chi^2_9 = 46.1$, $p <0.001$), but not *vice versa* (F:S and F:Y AIC =142.5; $\chi^2_9 = 4.83$, p = 0.85). This is likely to be an artefact of the timing of sampling, as no samples were collected in incubation in 2014. During incubation in 2015, samples comprised mostly rockcod, whereas in both years, samples collected during early chick-rearing were mostly of sprat and in 2014 were of kingclip. During late chick-rearing diet was more diverse, including hoki and rockcod in both years, southern blue whiting in 2014, and sprat in 2015 (Table 5.5; Figure 5.5).

South Georgia

Sixteen fish species were found in the 68 samples from Bird Island, with two species particularly common in both years: South Georgia icefish (*Pseudochaenichthys georgianus;* 48% and 42% FOO) and mackerel icefish (*Champsocephalus gunnari*; 44% and 34% FOO). Marbled rockcod (*Notothenia rossii;* 26% and 24% FOO), yellow-fin notothen (*Patagonotothen guntheri*; 11% and 17% FOO*)* and humped rockcod (*Gobionotothen* sp.; 11.1% and 17.1% FOO) were also common. In 2014, moray cod (*Muraenolepis* (*microps/orangiensis;* 14.8% FOO) was in >10% of samples, whereas in 2015 a large

proportion of samples included blackfin icefish (*Chaenocephalus aceratus*; 29% FOO) and southern driftfish (*Icichthys australis;* 27% FOO) (Table 5.6; Figure 5.5). There was an effect of year (Base model AIC=200.2, F:Y AIC =196.3; $\chi^2_{15} = 33.9$, $p < 0.01$) and breeding stage on fish consumed (F:S AIC =200.7; $\chi^2_{15} = 29.5$, p=0.01). However, although breeding stage was statistically significant, the base model excluding stage still provided a better fit to the data, even when both year and stage were included (F:Y and F:S AIC =207.3; $\chi^2_{15} = 18.9$, p=0.21; F:S and F:Y AIC =207.3; $\chi^2_{15} = 23.3$, p=0.08).

Iles Kerguelen

Eleven fish species were found in the 46 samples from Iles Kerguelen, with the main fish species grey rockcod (*Lepidonotothen squamifrons*; 53% and 56% FOO) and unicorn icefish (*Channichthys rhinoceratus;* 33% and 19% FOO) in both years. In 2014, the other common species were skates (*Bathyraja* sp.; 17% FOO) and moray cod (*Muraenolepis marmoratus/orangiensis*; 10% FOO), whereas in 2016, Patagonian toothfish (*Dissostichus eleginoides*) was the second most common item (44% FOO; Table 5.6, Figure 5.5). There were more samples with unicorn icefish during incubation than chick-rearing, whereas all the toothfish was consumed during chick-rearing. There was an effect of year (base model AIC=113.6, F:Y AIC =112.8; $\chi^2_{10} = 20.8$, $p = 0.03$) and breeding stage on the fish species consumed (F:S AIC =113.1; $\chi^2_{10} = 20.5$, $p<0.03$).

Macquarie Island

Sixteen species were found in the 36 samples from Macquarie Island (Table 5.6). In both years, samples mostly contained Antarctic horsefish (*Zanclorhynchus spinifer*; 65% and 70% FOO) and Magellanic rockcod (*Paranotothenia magellanica*; 31% and 10% FOO). In 2015, one unidentified species, likely from the family Bramidae, made up 20% of samples, although this may reflect the small sample size (n=10). Fish species composition did not differ between years (Base AIC= 177.4, F:Y AIC=190.6; $\chi^2_{15} = 16.8$, $p = 0.33$); the effect of breeding stage was of borderline statistical significance (F:S AIC =193.5; $\chi^2_{30} = 43.9$, p=0.05), but the base model excluding stage still provided a better fit to the data.

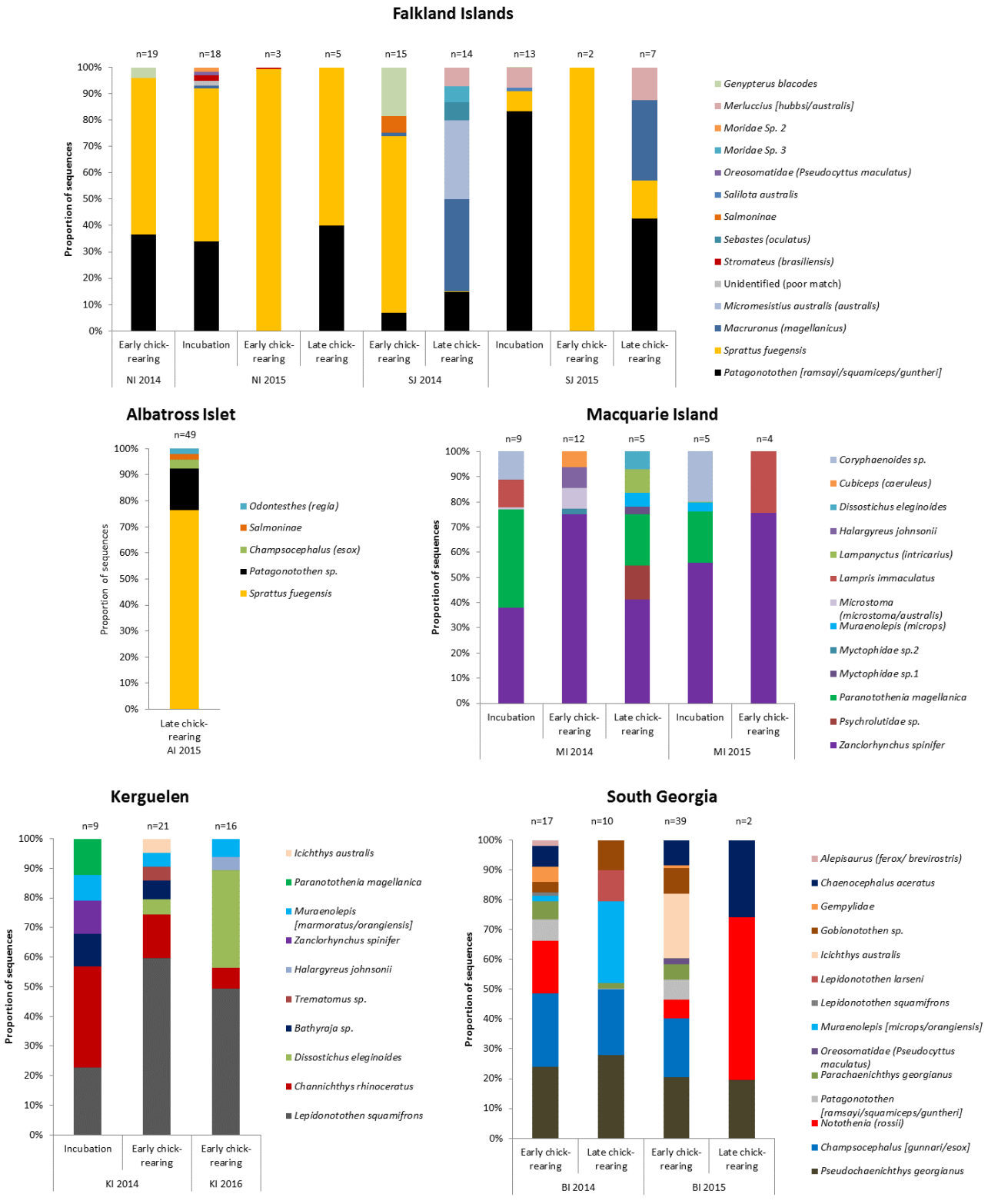

Figure 5.5 The proportion of fish sequences in the diet of black-browed albatrosses by breeding stage, site and year. Breeding stages were: incubation (October-mid December), early-chick rearing (mid-December-end of January), late-chick-rearing (February onwards). The single sample collected at Macquarie Island during late chick rearing in 2015 was excluded (the DNA sequences were all from the family Bramidae; genus unknown).

Table 5.4 Main fish prey at each sampling colony of black-browed albatrosses. Stars represent the frequency of occurrence (FOO) and relative read abundance (RRA) of amplified fish sequences: *10-25%, ** 25-50%, *** 50-75% and ****>75%. A coloured star is used when the FOO (blue) or RRA (red) were greater than the other measurement. Only species that occurred in more than one sample are included. Species in brackets are those where the genus could be confirmed but the species were either genetically similar [square brackets] or not all in Genbank (round brackets). Where only one species is in the bracket, this is the likely species given spatial distribution. Sites were Albatross Islet, Chile (AI); New Island (NI) and Steeple Jason Island (SJI), Falkland Islands; Bird Island, South Georgia (BI); Canyon des Sourcils Noirs, Iles Kerguelen (KI); and Macquarie Island (MI).

Family	Taxa	AI 2015	NI 2014	NI 2015	SJI 2014	SJI 2015	BI 2014	BI 2015	KI 2014	KI 2016	MI 2014	MI 2015
Bathydraconidae	*Parachaenichthys georgianus*						*					
Bramidae	Unidentified sp.											*
Centrolophidae	*Icichthys australis*							**				
Channichthyidae	*Chaenocephalus aceratus*[+]							**				
	*Champsocephalus [gunnari/esox]^*						**	**				
	Channichthys rhinoceratus								**	*		
	Pseudochaenichthys georgianus[+]						**	**				
Clupeidae	*Sprattus fuegensis*	****	***	***	***	**						
Congiopodidae	*Zanclorhynchus spinifer*										***	***
Gadidae	*Micromesistius australis^*[#]				**	*						
Merlucciidae	*Macruronus (magellanicus)^*[#]				**	*						
Muraenolepididae	*Muraenolepis [microps/orangiensis]*						*					
Myctophidae	*Lampanyctus (intricarius)*											*
Nototheniidae	*Dissostichus eleginoides^*[#]									**		
	Lepidonotothen larseni						*					
	Lepidonotothen squamifrons								***	***		
	Notothenia rossii[+]						**	**				
	Paranotothenia magellanica										**	
	Patagonotothen [tessellata/brevicauda]	**										
	Patagonotothen [ramsayi[#]*/squamiceps/guntheri^]*		***	**	**	***	*	*				
	Gobionotothen sp.						**	**				
Ophidiidae	*Genypterus blacodes^*				*							
Arhynchobatidae	*Bathyraja* sp.[+ #]								*			

^ target fishery species; [+]bycatch species, [#]not naturally accessible. NB: Only species that occurred in more than one sample are included.

Table 5.5 Fish prey of black-browed albatrosses at Albatross Islet (AI), Chile; and New Island (NI) and Steeple Jason Island (SJI), Falkland Islands. Number of samples (n), frequency of occurrence (FOO, %) and relative read abundance (RRA, %). Species in brackets are those where the genus could be confirmed but the species were either genetically similar [square brackets] or not all in Genbank (round brackets). Where only one species is in the bracket, this is the likely species given spatial distribution.

		AI 2015 (n=49)			NI 2014 (n=19)			NI 2015 (n=26)			SJI 2014 (n=29)			SJI 2015 (n=22)		
Family	**Taxa**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**
Atherinopsidae	*Odontesthes (regia)*	1	2	2												
Channichthyidae	*Champsocephalus [esox]*	3	6	3.5												
Clupeidae	*Sprattus fuegensis*	43	88	76	13	68.4	59.5	18	69.2	63.2	14	48.3	34.9	4	18.2	18.3
Emmelichthyidae	*Emmelichthys nitidus (cyanescens)*	1	2	0												
Gadidae	*Micromesistius australis (australis)*										6	20.7	14.4	1	4.5	0.1
Merlucciidae	*Macruronus (magellanicus)*							1	3.8	0.8	6	20.7	17.5	3	13.6	9.7
	Merluccius [hubbsi or *australis]*										2	6.9	3.4	2	9.1	8.5
Monacanthidae	Monacanthidae sp.	1	2	0.1												
Moridae	*Salilota australis*													1	4.5	0.7
	Moridae sp. 2							1	3.8	1.1						
	Moridae sp. 3										1	3.4	3			
Nototheniidae	*Patagonotothen [tessellata/brevicauda]*	14	29	16												
	Patagonotothen [ramsayi/squamiceps/guntheri]				10	52.6	36.5	9	34.6	31.2	5	17.2	10.7	14	63.6	62.8
Ophidiidae	*Genypterus blacodes*				1	5.3	3.9				3	10.3	9.6			
Oreosomatidae	Oreosomatidae *(Pseudocyttus maculatus)*							1	3.8	1						
Arhynchobatidae	*Bathyraja* sp.															
Salmonidae	Salmoninae sp. 1	1	2	2												
	Salmoninae sp. 2										1	3.4	3.2			
Scombridae	*Scomber* sp.	1	2	0.1												
Sebastidae	*Sebastes (oculatus)*										2	6.9	3.2			
Stromateidae	*Stromateus (brasiliensis)*							2	7.7	1.5						
Unmatched	Unmatched							2	7.7	1.2						

Table 5.6 Fish prey of black-browed albatrosses at Bird Island, South Georgia UK (BI); Iles Kerguelen, France (KI) and Macquarie Island, Australia (MI). Number of samples (n), frequency of occurrence (FOO) and relative read abundance (RRA). Species in brackets were those where the genus could be confirmed but the species were either genetically similar [square brackets] or not all on Genbank (round brackets). Where only one species is in the bracket, this is the likely species given spatial distribution.

		BI 2014 (n=27)			BI 2015 (n=41)			KI 2014 (n=30)			KI 2016 (n=16)			MI 2014 (n=26)			MI 2015 (n=10)		
Family	**Taxa**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**	**n**	**FOO**	**RRA**
Alepisauridae	*Alepisaurus (ferox/ brevirostris)*	1	3.7	1.2															
Anotopteridae	*Anotopterus vorax*	1	3.7	0.1	1	2.4	0.1												
Bathydraconidae	*Parachaenichthys georgianus*	3	11.1	4.4	4	9.8	4.8												
Bramidae	Unidentified sp.																2	20	10
Centrolophidae	*Icichthys australis*				11	26.8	20.4	1	3.3	3.3									
Channichthyidae	*Chaenocephalus aceratus*	2	7.4	4.4	12	29.3	9.3												
	Champsocephalus [gunnari/esox]	12	44.4	23.5	14	34.1	18.7												
	Channichthys rhinoceratus							10	33.3	20.6	3	18.8	6.9						
	Pseudochaenichthys georgianus	13	48.1	25.4	17	41.5	20.2												
Congiopodidae	*Zanclorhynchus spinifer*							1	3.3	3.3				17	65.4	55.7	7	70	57.3
Gempylidae	Unidentified sp.	1	3.7	3.3	1	2.4	0.8												
Harpagiferidae	*Harpagifer (macquariensis/georgianus)*													1	3.8	0.1			
Lampridae	*Lampris immaculatus*													1	3.8	3.8	1	10	9.8
Macrouridae	*Coryphaenoides* sp.													1	3.8	3.8	1	10	10
Microstomatidae	*Microstoma (microstoma/australis)*													2	7.7	4.2			
Moridae	*Halargyreus johnsonii*										1	6.3	4.4	1	3.8	3.8			
Muraenolepididae	*Muraenolepis [marmorata/orangiensis]*							3	10	5.9	1	6.3	6.2						
	Muraenolepis [microps/orangiensis]	4	14.8	11.4															
	Muraenolepis sp.													1	3.8	1	1	10	1.7
Myctophidae	*Lampanyctus (intricarius)*													1	3.8	1.8	1	10	0.1
	Myctophidae sp.1													1	3.8	0.6			
	Myctophidae sp.2													1	3.8	1			
Nomeidae	*Cubiceps (caeruleus)*													1	3.8	2.9			

Nototheniidae	*Dissostichus eleginoides*							2	6.7	3.5	7	43.8	32.9	1	3.8	1.3			
	Lepidonotothen larseni	3	11.1	3.8															
	Lepidonotothen squamifrons	1	3.7	0.7	1	2.4	0.3	16	53.3	48.5	9	56.3	49.4						
	Notothenia rossii	7	25.9	11.1	10	24.4	8.7												
	Paranotothenia magellanica							2	6.7	3.7				8	30.8	17.4	1	10	10.1
	Patagonotothen (ramsayi/squamiceps/guntheri)	3	11.1	4.6	7	17.1	6.4												
	Trematomus sp.							1	3.3	3.3									
	Gobionotothen (gibberifrons/marionensis)	3	11.1	6	7	17.1	8.2												
Oreosomatidae	Oreosomatidae *(Pseudocyttus maculatus)*				1	2.4	1.7												
Paralepididae	*Magnisudis (atlantica/prionosa)*				1	2.4	0.2												
Psychrolutidae	Psychrolutidae sp.													1	3.8	2.6			
Arhynchobatidae	*Bathyraja* sp.							5	16.7	7.7									

5.4.2 Overlaps between commercial fishery species and BBA prey

Longline fisheries

Diets of BBA from New Island and Steeple Jason Island did not include any target or bycatch species from longline fisheries operating in the Falkland Islands FICZ/FOCZ. At Iles Kerguelen, diet samples included DNA from the target species, Patagonian toothfish, in January of both years (with much higher proportions in 2016; Tables 6 and 7) and a bycaught group, skates, in December/January 2014/15 (Table 5.7, Figure 5.6). Bait fish, *Scomber scombrus* also appeared in samples, but occurred infrequently (< 2% of sample sequences). This is a northern hemisphere species used as baits in longline fishing, and is therefore only available to BBA from fisheries. In the Kerguelen EEZ, the amount of toothfish discarded was lowest in November and December 2013 (0.19t and 0.18t respectively) and highest in January (1.6 t in 2014 and 2.9 t in 2016). More skates were discarded in December and January 2013/14 (0.3t in November, 5.3t in December and 9.4t in January 2013/14; and 2t in January 2016), which matched with the relative FOO in the diet in the two years.

Trawl fisheries

The trawl fisheries operating in the Falkland FICZ/FOCZ target eight fish species (Table 5.3). No bycatch species made up more than 1% of the reported catch. Fishery target species were found in the diet at both sites in each year (Table 5.7, Figure 5.6). At New Island, the main fishery target species in the diet was rockcod (91% of those samples with a target species); one sample also contained hoki, and one was of kingclip only. At Steeple Jason Island, BBAs consumed five target species in 2014 (rockcod, hake, hoki, southern blue whiting and kingclip), whereas samples included three target species in 2015 (rockcod, hoki and hake; Table 5.6, Figure 5.7). The number of samples with fishery target species was lower during early chick rearing (December/January) than either incubation (October-November) or late chick rearing (February-March; Figure 5.7a). This corresponded to the relative catch in the fishery, particularly during January when it was not operating (Figure 5.7a). The main catch species in the fishery was rockcod during incubation, and hoki in late chick rearing (Figure 5.7c). The cluster analysis showed four distinct clusters, with one highlighting the similarity between fishery catch and fish prey of BBA during December 2013 at New Island and October 2014 at both sites, and between fishery catch during March and fish prey of BBA at Steeple Jason (Figure 5.7d).

At South Georgia, the fishery target species (mackerel icefish) and four bycatch species (South Georgian icefish, yellow-fin notothen, grey rockcod and marbled rockcod) were all recorded in the diets of BBA (Table 5.6), and in a substantial proportion of the samples in both years (Table 5.7,

Figure 5.6). The amount of target and bycatch fish species in the diet of BBA did not correspond to the relative catch rates in the fishery. During the sampling period the fishery caught very little mackerel icefish during January 2014 (65 kg), and only 3 tonnes during February 2014, with one tonne of South Georgia icefish as bycatch. Over the same period in 2015, the fishery caught 133 tonnes of mackerel icefish in January, and 144 tonnes in February, with 70 and 51 tonnes of yellow-fin notothen bycaught in each month, respectively. Other bycatch included one tonne of South Georgia icefish, 2 tonnes of grey rockcod and 4 tonnes of marbled rockcod, all of which were caught during the 2015 South Georgia groundfish survey.

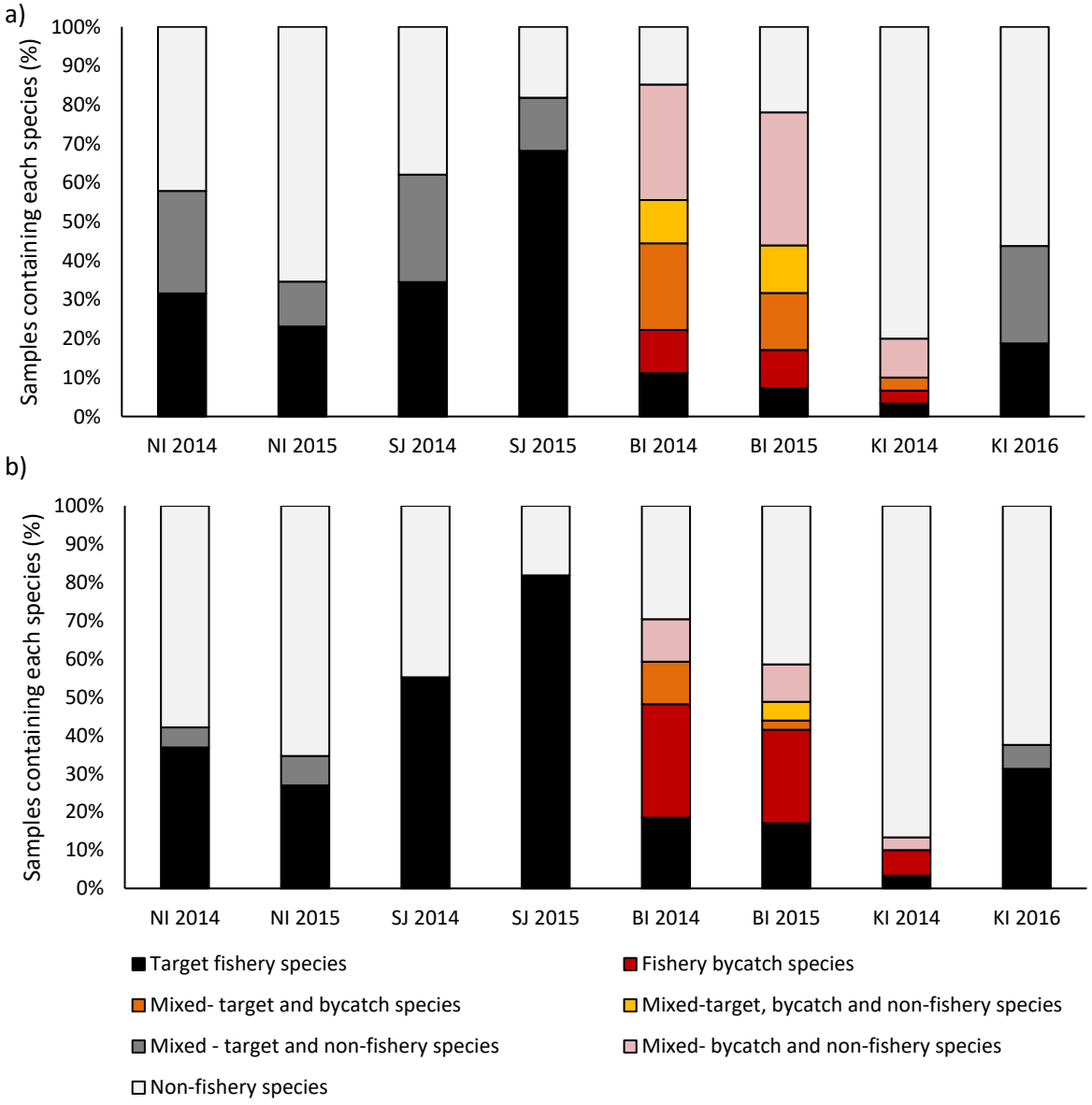

Figure 5.6 The proportion of samples that contained target, bycatch or non-fishery species as either a) >1% of sequences in a sample, or b) >75% of the sequences in a sample. Samples with <75% of sequences in any category were considered to be mixed. Bycatch species each constituted >1% of the total catch. Sites are New Island (NI) and Steeple Jason Island (SJI), Falkland Islands; Bird Island, South Georgia (BI) and Canyon des Sourcils Noirs, Iles Kerguelen (KI).

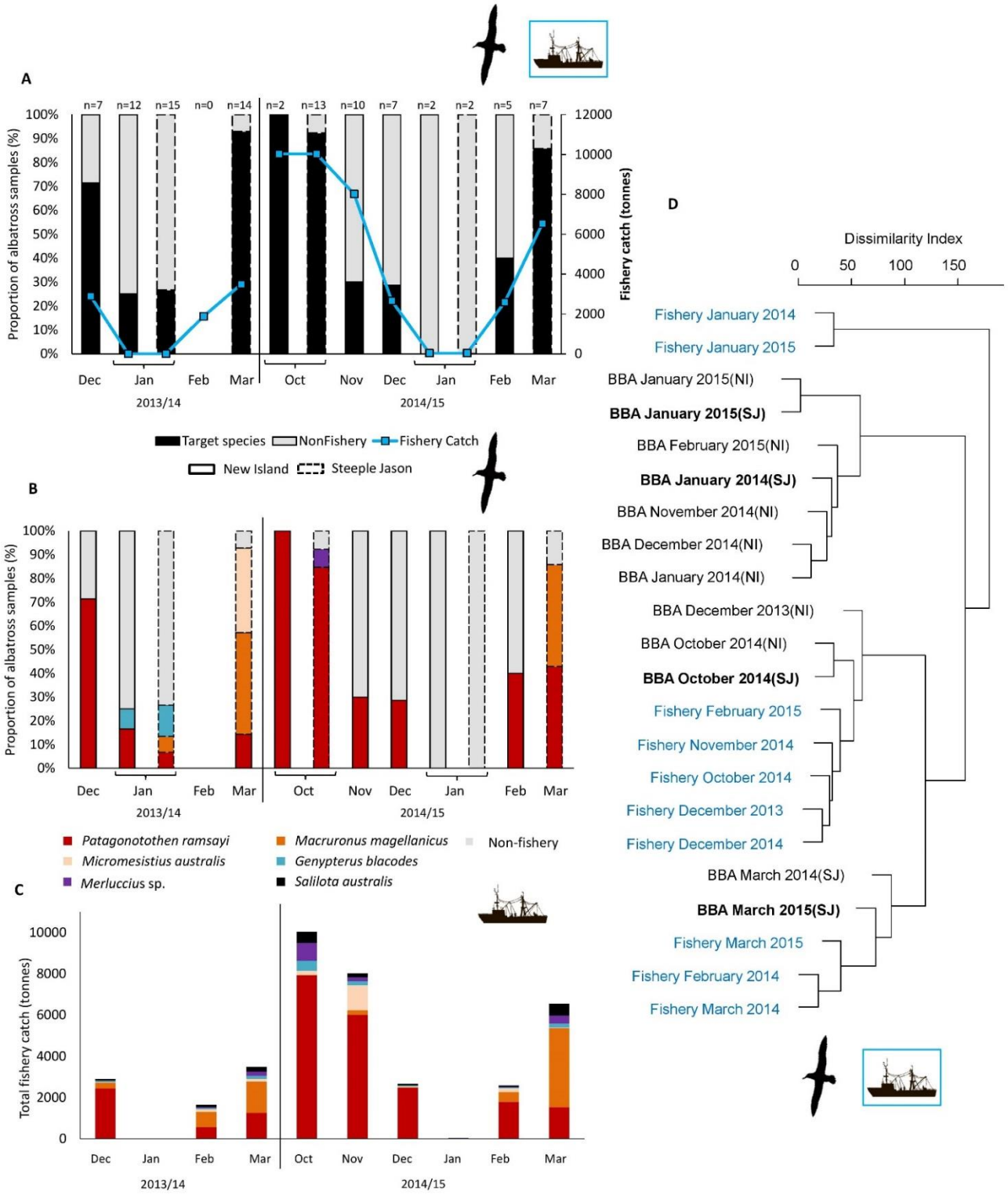

Figure 5.7 Comparison between back-browed albatross fish prey and fishery catch amounts at the Falkland Islands by month from December 2013-March 2014 (excluding February) and October 2014 to March 2015. Solid borders represent New Island; dashed borders represent Steeple Jason. A) Scats with or without fishery target species (black and grey bars respectively), compared to the total catch in the fishery (blue line). B) The proportion of scat samples containing each of the target species. C) Total catches in the fishery by species. D) The hierarchical clustering of albatross diet and fishery catch data by month, based on the proportion of sequences (RRA) and proportion of catch. Clusters were based on dissimilarity indices calculated with the Manhattan method, and hierarchical clustering was constructed using the average agglomeration method

(note low sample sizes during January 2014 and 2015). As food DNA may persist in scats for several days (Deagle et al. 2010), there may be some carry-over of prey caught in the previous month if samples were collected early in the month, which was the case in January of both years.

5.4.3 Breeding success and use of discards

The proportion of sampled birds that had consumed discards was estimated to range from zero at Bird Island to 60% at Steeple Jason. This is based on the conservative assumption that any species which was also available naturally to albatrosses was not considered to have been obtained as a discard (Table 5.7). Breeding success (chicks fledged/eggs laid) during the years that diet samples were collected ranged from zero at Albatross Islet to 84% at New Island. There was a positive correlation between the proportion of samples that contained discarded fish, and breeding success ($r = 0.81$, $p < 0.001$; Figure 5.8).

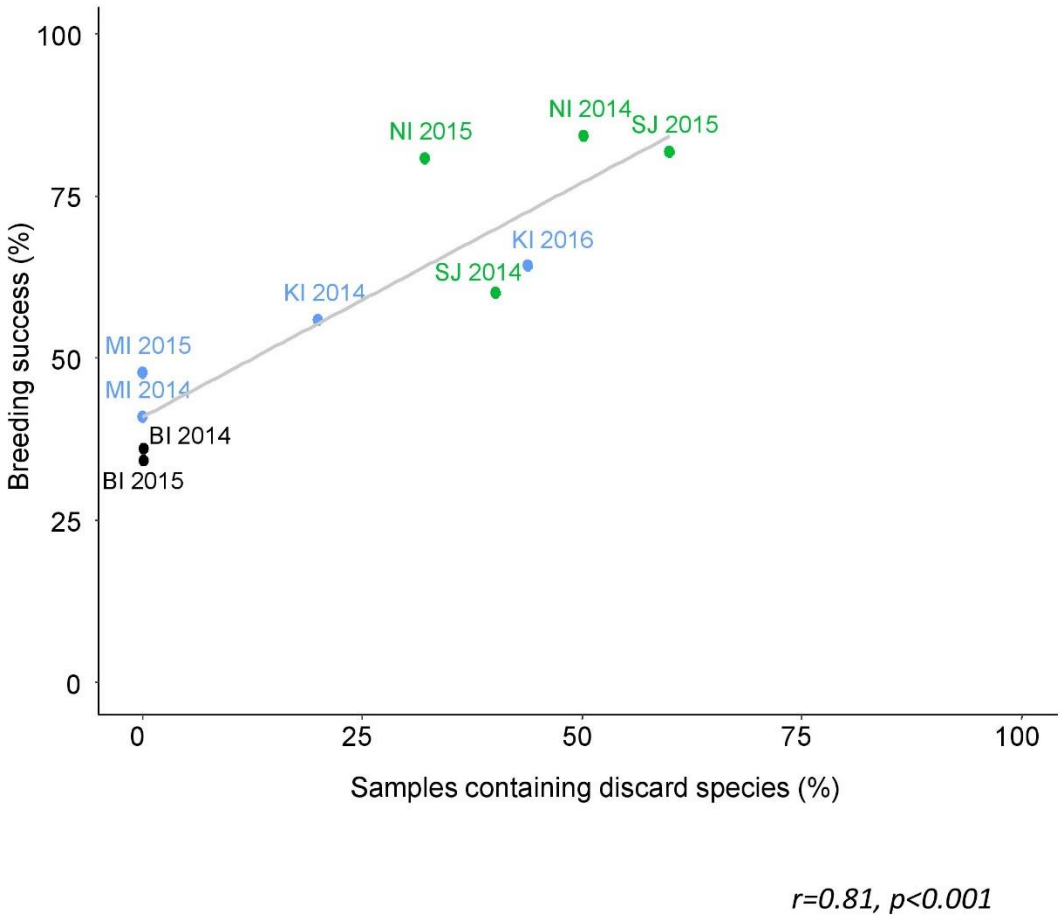

Figure 5.8: The proportion of scat samples from black-browed albatrosses that contained discard species in relation to breeding success for that site and year. The colours corresponds to the current population trend: green – increasing; blue – stable; black - declining (ACAP 2010, Wolfaardt 2013, Poncet et al. 2017). These are minimum estimates of discard occurrence, including only the species that are not known to be naturally accessible to albatrosses (Appendix 5.2). Albatross Islet suffered complete breeding success failure due to mammalian predation and was therefore excluded from the analysis.

Table 5.7 The proportion of scat samples that contained DNA from target and bycaught species in commercial fisheries operating in adjacent waters during the study. These are the proportion of samples that amplified with the 16S_Fish primer (^), except the final two columns which is the proportion of all samples that contained food DNA amplified with the 18S_SSU primers (*). All listed fishery bycatch species constituted >1% of the total catch. Inaccessible fish species - those that occur below the dive depth of albatrosses; includes skates, toothfish, southern blue whiting, rock cod and hoki (Appendix 5.2).

Site	Year	Proportion of fish samples with fishery target species^	Proportion of fish samples with fishery bycatch species^	Proportion of fish samples containing any fishery species^	Proportion of fish samples with inaccessible species^	Proportion of all samples containing fishery species*	Proportion all samples with inaccessible species*
TRAWL FISHERY							
New Island	2013/14	57.9	0	57.9	52.6	55.3	50.2
	2014/15	34.6	0	34.6	34.6	32.1	32.1
Steeple Jason Island	2013/14	62.1	0	62.1	55.2	45.3	40.2
	2014/15	81.8	0	81.8	77.3	63.4	59.9
Bird Island	2013/14	44.4	74.1	85.2	0	77.4	0
	2014/15	34.1	70.7	78.1	0	72.5	0
LONG-LINE FISHERY							
New Island	2013/14	0	0	0	0	0	0
	2014/15	0	0	0	0	0	0
Steeple Jason Island	2013/14	0	0	0	0	0	0
	2014/15	0	0	0	0	0	0
Iles Kerguelen	2013/14	6.7	16.7	20.0	20	20.0	20
	2015/16	43.8	0	43.8	43.8	43.8	43.8

5.5 Discussion

This is the first study to use DNA metabarcoding to identify the fish prey diversity of seabird and use this to evaluate the occurrence of fishery discards in the diet across a broad geographic scale. This technique enabled us to identify an extensive diversity of fish in the diet of BBA, including a similar number of species and families as that recorded in all previous published studies for this species combined (31 families and 52 species). We also detected more fish species on average at each sampling site than in the conventional studies based on otoliths and similar numbers to studies using multiple body parts (Appendix 5.1). There was a clear overlap between the species targeted by fisheries operating in adjacent waters, and the diet of BBA at the local colony. This was most evident at the Falkland Islands and Iles Kerguelen where the higher catch rates of target and bycatch species (or the amount discarded, where known) in a particular year corresponded with the relative occurrence in the diet. Our data also highlighted regions, such as South Georgia, where BBA diet overlapped with fishery target species, but the birds likely obtained the fish naturally. In this situation, there is the potential for resource competition but no reason to assume direct interaction with vessels or incidental mortality of foraging adults.

5.5.1 Amplification success

The number of samples that contained food DNA in this study varied between sites and in some cases were quite low. There are numerous factors that can affect the amplification of food DNA including the primers/markers chosen, whether blocking primers were used, sample selection, timing during the breeding season and experience of the field personnel. We chose the combination of the universal metazoan marker (18S) and group specific markers (16S) to get a broad picture of the diet at the population level and specific information on the fish species consumed. Universal metazoan markers are useful dietary markers as they amplify DNA from all eukaryotes, which enables all possible prey groups to be identified. However, they also amplify non-food DNA such as plant, parasite and consumer DNA (McInnes et al. 2017a). A consumer blocking primer can increase the detection of food DNA (Vestheim and Jarman 2008), but was not used in this study as they may inadvertently block similar groups such as other vertebrates like fish (Piñol et al. 2015). This likely reduced the samples size, but provided more reliable results from higher quality samples containing more food DNA. During our study, fresh samples were targeted and the inadvertent collection of dirt and vegetation was minimised where possible. However, with such a large study across a range of environmental conditions this was not always possible. In addition, many samples collected during incubation had little food DNA due to birds fasting. Subsequent to the data collection for this study,

optimised scat collection protocols for DNA dietary metabarcoding have been developed that will hopefully improve the amount of DNA detected in future studies allowing diet data to be collected during all breeding stages (McInnes et al. 2017a).

5.5.2 Fish prey diversity

The fish prey consumed by BBAs varied considerably across their breeding range. Species in the family Nototheniidae were common in scat samples from the sub-Antarctic sites, as were icefish (Channichthyidae) and horsefish (Congiopodidae). However, aside from the genus *Patagonotothen*, there were no nototheniids found in the samples from the Falkland Islands and Chile, whereas sprat (Clupeidae) was common. Of those species that contributed greater than 10% of the sequences/samples at any site, 80% of these fish species were likely to be obtained naturally, as they are known to occur at depths accessible to albatrosses (maximum 4.5m; Prince and Huin 1994). The remaining species are not known to occur in waters shallower than 4.5m and are hence likely to be obtained as discards.

This study detected several species that were not identified, or were very uncommon, in the diet of BBA in previous studies, particularly the Fueguian sprat, Antarctic horsefish and southern driftfish. This was the first study of fish in the diet of BBA at Albatross Islet, and the first published study of BBA diet at Macquarie Island, which may explain some of these discoveries. Sprat was not recorded previously in the diet of BBA at any site (Appendix 5.1), despite being the most common item in our study at the Falkland Islands and Chile. There was an unidentified clupeid in the diet at Diego Ramirez in 2001 and 2002 (Arata and Xavier 2003), and a small unidentified fish at New Island in 1987 that made up 80% of the fish prey (Thompson 1992), which may have been sprat. This species has a high biomass across the southern Patagonian shelf as far as the Magellan Strait (Sánchez et al. 1995), Chilean channel waters (Diez et al. 2012) and around the Falkland Islands (Agnew 2002), and is common in the diet of other seabirds and marine mammals in the region (Thompson 1993, Baylis et al. 2014, Handley et al. 2016). There is also a sprat hotspot to the west of the Falkland Islands, close to both Steeple Jason and New Island, which may explain the prevalence in the diet at these sites (Gras et al. 2017).

Antarctic horsefish was the main fish species consumed at Macquarie Island and is endemic to the Macquarie and Kerguelen plateaus (Duhamel et al. 2014). Antarctic horsefish has been detected previously, but only in low frequency in BBA diets at Iles Kerguelen (Cherel et al. 2000b, 2002). Very little is known about the abundance of horsefish or other fish around Macquarie Island. Horsefish

have been detected in the diet of gentoo penguins (*Pygoscelis papua;* 39% FOO) and itinerant New Zealand sea lions at Macquarie Island (*Phocarctos hookeri*; 63% FOO) (Robinson and Hindell 1996, McMahon et al. 1999); however the majority of fish consumed by other seabirds and seals are myctophids, and to a lesser extent nototheniids (Goldsworthy et al. 2001).

The southern driftfish was detected in a quarter of samples at Bird Island in 2015 and one sample at Iles Kerguelen. It has only been recorded once in the diet of BBA at South Georgia, in 1986 (Reid et al. 1996, Croxall et al. 1997), and rarely in the diet of other seabirds (Croxall et al. 1995, Catard et al. 2000), though has been detected more commonly in seal diets (Guinet et al. 2001, Lea et al. 2008). Southern driftfish have a circumpolar distribution (Gon and Heemstra 1990), although are rarely caught during trawls in the Scotia Sea (Collins et al. 2012) and none were recorded during a groundfish survey in January 2015, at the time when the scat samples were obtained (Belchier et al. 2015). It is surprising given our results that only one sample was detected in 20 years of conventional diet studies at South Georgia (British Antarctic Survey unpublished data). There are a few possible explanations: most of the previous studies were later in the season (February onwards) and represent chick diet, whereas our samples were from adults; alternatively, driftfish may be consumed as larvae and therefore the hardparts may be undetectable in stomach contents.

The other main fish prey at Bird Island and Iles Kerguelen were similar to the previous studies at each site (Appendix 5.1). At South Georgia, mackerel icefish are common prey (Prince 1980, Reid et al. 1996, Croxall et al. 1997, Croxall et al. 1999, Xavier et al. 2003a). However, the diversity of fish in our study was much higher than in previous studies at Bird Island using only otoliths, which identified ten fish species overall, and less than five in any year (Appendix 5.1). In comparison, we detected 16 species using DNA metabarcoding, with 13 in each year. Some of this diversity could relate to secondary ingestion; however, all of the species that contributed >10% of the diets (n=8 and n=7) were the sole prey item in at least one sample, suggesting they were the primary prey. At Iles Kerguelen, grey rockcod and unicorn icefish are two of the most abundant fish species in the Kerguelen EEZ (Duhamel and Hautecoeur 2009) and were common in the diet of BBA at Canyon des Sourcils Noirs in a previous study (Cherel et al. 2000b). When sample size differences were taken into account, the fish diversity was similar to previous studies at Iles Kerguelen where otoliths, bones and vertebrae were used (Cherel et al. 2000b). In our study, there were some fish species identified in just one sample that are not usually found at those sites, such as *Trematomus* sp. at Iles Kerguelen. These could have originated from scats produced by juvenile or non-breeding birds, or as

residual DNA from previous foraging trips far from the islands. For these reasons, we focused on fish species present in at least 10% of samples.

5.5.3 Overlaps between commercial fisheries and albatross diet

There were five fish species detected in the scats that were unlikely to be naturally accessible to BBAs during the sampling period due to the known depth profile of fish. These included skates and Patagonian toothfish at Iles Kerguelen, and rockcod, hoki and southern blue whiting at the Falkland Islands. These species were present in fishery catches from the same time period, suggesting vessels were the likely source. At Iles Kerguelen, Patagonian toothfish and skates have no developmental stage where they have been observed at an accessible depth to albatross (Appendix 5.2). Skates are demersal and the closest to the surface that toothfish have been recorded is during their larval stage (~50m depth), during winter and spring at Iles Kerguelen (Loeb et al. 1993, Mori et al. 2016). Patagonian toothfish were the most common fish in previous BBA dietary studies at Iles Nuageuses in 1994, and second most common at Canyon des Sourcils Noirs in 1994 (Cherel et al. 2000b, 2002). These studies and others on wandering albatross (*Diomedea exulans*) suggest that albatross can consume Patagonian toothfish naturally (Weimerskirch et al. 1997b), but how they obtain demersal prey is largely unknown (Cherel et al. 2000b). It is possible that albatrosses scavenge prey brought up by deep-diving predators such as seals or whales (Sakamoto et al. 2009). In our study, the occurrence of Patagonian toothfish in the diet of birds from Iles Kerguelen did increase with an increase in discards, however, the amount of discarded toothfish from the fishery was low relative to the large albatross population. This result suggests that albatross may also be consuming Patagonian toothfish as natural prey as well as fishery discards during this study in unknown respective proportions. Although seabird bycatch rates in this fishery were very high in the 1990s and early 2000s (Delord et al. 2005), no albatross mortalities have been reported in recent years (CCAMLR 2014a). This reflects the adoption of mitigation measures which include night setting, streamer lines, retention of offal during setting and fast hook sink rates (CCAMLR 2014b). Discarding is still permitted in the Kerguelen toothfish fishery, which is still of concern. Discards increase vessel attractiveness and it is difficult to ensure mitigation is 100% effective for the smaller, more manoeuvrable, deeper-diving species, particularly those such as white-chinned petrels (*Procellaria aequinoctialis*) which, unlike albatrosses, scavenge behind vessels in large numbers during darkness (Phillips et al. 2016). Moreover, individual birds will associate vessels with food, which is problematic if they overlap with fisheries under a different jurisdiction where there is poor compliance with seabird bycatch mitigation. Indeed, wandering albatrosses, which habitually follow vessels, may alter their flight path from 30km away to approach a fishing vessel (Collet et al. 2015).

At the Falkland Islands, the frequency of rockcod and hoki in the diet corresponded to the relative fishery catches of these species, suggesting they were likely obtained as discards. Although this correlation could also reflect availability of fish stocks, these species are not known to be naturally accessible to albatross. Occurrence varied between sites and breeding stages, and was lowest during early chick rearing, which is consistent with the previous stable isotope study which found that pelagic fish were more common than demersal species (Granadeiro et al. 2013). During early chick-rearing, the fishery catch was zero to low, and therefore there was limited opportunity to exploit discards. However, during incubation and late chick rearing, the frequency of target fishery species in the albatrosses' diet was much higher. The occurrence of fishery species in BBA samples was greater at Steeple Jason than New Island, which is 70 km further south. This may be an artefact of the sampling month, given differences in timing; however, this does not explain all of the variation. The samples collected in the same month (e.g. January) were comparable, but no trawl fishery was operating. The few samples with fishery target species at New Island in November, when catch rates were relatively high, does not seem to match the trend at Steeple Jason for the preceding month. Previous studies at the Falklands also found more offal and discards in the diet of BBA at Steeple Jason than New Island, however, as there were few heads and therefore otoliths, the fish species could not be identified (Thompson 1992). In the western part of the FICZ, there are two types of fishing grounds: one is to the northwest of Steeple Jason where trawlers target rockcod and one in deeper waters (>200 m) to the west-southwest which targets primarily hoki and southern blue whiting. Previous tracking studies found that Steeple Jason birds were more likely to attend vessels even when the distance to the fishing ground was similar for each colony (Granadeiro et al. 2011). Further research is needed to understand this observation.

The consumption of fishery discards by black-browed albatrosses at both sites in the Falklands puts birds at risk of incidental mortality. An estimated 800 BBAs are killed annually in Falkland Island trawl fisheries (Kuepfer 2015). Although use of paired streamer lines is compulsory on all vessels, continuous discarding is still permitted (Quintin and Pompert 2014). At the time of this study, the fishing fleet had limited capacity to retain offal on-board or process this into fishmeal; however, it has been recommended that any new vessels entering the fishery should have capabilities for more effective waste management (Sancho 2009). Strict discard policies employed by trawl vessels operating in waters within the jurisdiction of CCAMLR have minimised exposure of birds to warp cables by retaining discards on-board until after shooting or hauling of fishing gear; consequently, the occurrence of incidental mortality is close to zero (CCAMLR 2014b). Implementation of improved

discard management measures around the Falkland Islands will be essential to reduce incidental mortality in trawl fisheries in the future (Abraham et al. 2009, Pierre et al. 2012).

5.5.4 Competition with fisheries and reliance on fishery discards

South Georgian and mackerel icefishes were the two most common fish consumed by BBA at South Georgia in both years. Although mackerel icefish is targeted by the fishery, and South Georgia icefish is bycaught, the BBA at Bird Island were likely to have obtained these species naturally. Very few fish were caught by the fishery during the diet sampling period of 2014 and they are known to occur at an accessible depth to albatross. Five other common prey species of BBAs at South Georgia are also caught in the icefish fishery, although bycatch limits are set by CCAMLR (South Georgia icefish, marbled rockcod, yellow-fin notothen, humped rockcod, blackfin icefish). Mackerel icefish was the most common fish in the diet of BBA at South Georgia from 1996-2000 (Xavier et al. 2003a) and in more recent years (British Antarctic Survey unpublished data). Icefish and BBA are both krill predators, and in years of low krill availability, icefish are likely to provide a valuable alternate food source for albatrosses (Reid et al. 1996). The BBA population at South Georgia is declining, and although this appears to be due mainly to incidental mortality during the non-breeding period (Poncet et al. 2017), their breeding success is also lower than conspecifics in the Indian Ocean (Nevoux et al. 2010). During our study, the proportion of krill in the diet was low (Figure 5.3), and over the last 20 years of conventional sampling (in mid-late chick rearing), krill has contributed <20% of the diet in only four years, two of which were 2014 and 2015 (18.5% and 5.6%, respectively; British Antarctic Survey unpublished data). Given the decline in krill, and high consumption by BBAs of species that are also targeted or bycaught in the icefish fishery, continued monitoring and evaluation of potential competition for resources is particularly important at this breeding site.

Another area of potential resource competition is off Chile, where there is currently a sprat fishery between 41-45°S, with annual catch limits of 26,000 tonnes (Leal et al. 2013). There was a proposal to expand this fishery into Chilean channel waters, where it would be likely to overlap with the foraging areas of BBAs from Chilean colonies. Given the importance of sprat in diets, any expansion of the fishery should consider the resource requirements of other marine species, including BBA, especially at the Albatross Islet colony where the foraging range is restricted (Arata et al. 2014). Globally, a third of fish stocks are fished at unsustainable levels (FAO 2016), and fisheries are fishing down the food web (Pauly et al. 1998), including smaller fish species like sprat (Leal et al. 2013).

Although competition with commercial fisheries could have a negative impact on albatrosses by reducing available prey, discards from fisheries can provide a supplementary food source (Bugoni et al. 2010; this study). In our study, breeding success was higher at colonies which had a greater occurrence of fishery discards in the diet samples. At the Falkland Islands where the occurrence of discards was high, the BBA population is increasing (Wolfaardt 2013). Population increases at Chilean colonies have been attributed to a reduction in bird bycatch in longline fisheries (Robertson et al. 2017). However, high breeding success and a population increase could also reflect greater discard availability. Conversely, at Macquarie Island, where there is no local fishery operating during the breeding season, breeding success of BBAs was lower and the population is stable (ACAP 2010), and at South Georgia, where the icefish fishery is small and provides few discards, BBAs have the lowest breeding success and the population is declining (Poncet et al. 2017). Many factors can impact breeding success, and a snapshot of diet over two years is not definitive. For example, the total failure at Albatross Islet was likely due to predation of eggs and chicks by American mink (*Neovison vison;* WCS unpublished data). However, availability of discards can influence seabird population trends (Foster et al. 2017), and DNA metabarcoding provides a means of further investigation.

Discards create an unnatural food-web structure, and if they are of low nutritional quality, there may be impacts on growth, breeding success and survival (Rosen and Trites 2000, Grémillet et al. 2008). For BBA, the increasing population trend and high breeding success at sites where discards were common suggests that these were not nutritionally poor. However, discards could be sustaining an artificially high population and their removal might increase inter and intra-specific competition for available resources. Indeed, the European Union is phasing out the practice of discarding bycatch species and offal from 2015-2019, and there are concerns about the negative consequences for scavenging seabirds (Bicknell et al. 2013). Southern blue whiting was the main prey targeted by trawl fisheries around the Falklands Islands up until 2006, at which point the stock collapsed, and rockcod (*Patagonotothen ramsayi*) increased rapidly (Laptikhovsky et al. 2013). Rockcod is now the main target of the trawl fishery and one of the most common fish in the diet of BBAs at the Falklands during this study. Recent rockcod stock assessments indicate that this species is also beginning to decline (Gras et al. 2017). Monitoring the impact on BBA breeding success and their ability to switch to other resources will be important for assessing the degree to which they have been relying on discards. Similarly, improved discard management in the local trawl fisheries may have implications for the BBA population, particularly in the short-term, and any negative effects might be

exacerbated by other threats such as climate change, habitat degradation, introduced pests or disease, which affect many albatrosses globally (Phillips et al. 2016).

5.5.5 Conclusions

This circumpolar study has revealed extensive fish diversity in the diet of BBA using DNA metabarcoding. Many of the fish species in the diet are not known to be naturally available to albatrosses, and were likely obtained by scavenging on discards (non-target fish, processing waste or used longline bait) from fisheries operating adjacent to the colony. Consumption of discards by black-browed albatrosses was detected from the Falkland Islands trawl fishery during incubation and late chick-rearing and from the Iles Kerguelen longline fishery during brood-guard. Our study indicates that improvements in discard management to reduce the attractiveness of vessels and hence incidental mortality of seabirds is likely to have major implications for some albatross populations. DNA metabarcoding of scat samples provides a non-invasive mechanism for quantifying and evaluating the level of interaction between seabirds and fisheries through identification of target and non-target fish, as well as the presence of baits. This provides an avenue for assessing compliance of fisheries with discard policies, and the effects on the level of interaction with scavenging seabirds.

5.6 Acknowledgements

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5.7 Data accessibility

Fish sequences not currently of Genbank were added including: *Sprattus fuegensis, Genypterus blacodes, Iluocoetes fimbriatus, Salilota australis, Icichthys australis, Anotopterus vorax, Halargyreus johnsonii* (GenBank accession numbers MF346066-074).

5.8 Appendices

Appendix 5.1 – Data from previous Black browed albatross diet studies

Location	Reference	Family	*Species*	Year	Proportion of	Method	% FOO	% n	% mass
Kerguelen	Cherel et al 2000	Achiropsettidae	*Mancopsetta maculata*	1994, 1995	Regurgitate	Regurgitate	0.9	0.1	0.3
Southern Brazil	Colabuono and Vooren 2007	Ariidae	*Unidentified Ariidae*	1994-2004	Whole stomach	Whole stomach	2	1	1
Southern Brazil	Colabuono and Vooren 2007	Batrachoididae	*Porichthys porosissimus*	1994-2004	Whole stomach	Whole stomach	4	1	2
South Georgia	Croxall et al 1997	Centrolophidae	*Icichthys australis*	1986	Regurgitate	Regurgitate	2.3	5.3*	11.7
Kerguelen	Cherel et al 2000	Channichthyidae	*Champsocephalus gunnari*	1994, 1995	Regurgitate	Regurgitate	10.5	2.3	2.1
Kerguelen	Cherel et al 2000	Channichthyidae	*Channichthys rhinoceratus*	1994, 1995	Regurgitate	Regurgitate	35.1	5.6	16.9
Kerguelen	Cherel et al 2000	Channichthyidae	*Unidentified Channichthyidae*	1994, 1995	Regurgitate	Regurgitate	2.6	0.4	0.6
Iles Nuageuses	Cherel et al 2002	Channichthyidae	*Champsocephalus gunnari*	1994	Regurgitate	Regurgitate	2.9	0.9	0.3
Iles Nuageuses	Cherel et al 2002	Channichthyidae	*Channichthys rhinoceratus*	1994	Regurgitate	Regurgitate	11.4	3.5	6.1
South Georgia	Prince 1980	Channichthyidae	*Pseudochaenichthys georgianus*	1976		Regurgitate			
South Georgia	Prince 1980	Channichthyidae	*Pseudochaenichthys georgianus*	1976		regurgitate			
South Georgia	Xavier et al 2003	Channichthyidae	*Champsocephalus gunnari*	1997	Regurgitate	Regurgitate	18	58	13
South Georgia	Xavier et al 2003	Channichthyidae	*Champsocephalus gunnari*	1998	Regurgitate	Regurgitate			
South Georgia	Xavier et al 2003	Channichthyidae	*Champsocephalus gunnari*	1999	Regurgitate	Regurgitate	43	54	18
South Georgia	Xavier et al 2003	Channichthyidae	*Champsocephalus gunnari*	2000	Regurgitate	Regurgitate	21	20	8
South Georgia	Croxall et al 1999	Channichthyidae	*Champsocephalus gunnari*	1994	Regurgitate	Regurgitate	2.6	11.8*	8.4
South Georgia	Croxall et al 1999	Channichthyidae	*Pseudochaenichthys georgianus*	1994	Regurgitate	Regurgitate	10.5	23.5*	41.2
Diego ramirez	Arata and Xavier 2003	Clupeidae	*Clupeidae* sp.	2001	Regurgitate	Regurgitate	4.7	0.5	0.1
Diego ramirez	Arata and Xavier 2003	Clupeidae	*Clupeidae* sp.	2002	Regurgitate	Regurgitate	8	1.4	0.2
Kerguelen	Cherel et al 2000	Congiopodidae	*Zanclorhynchus spinifer*	1994, 1995	Regurgitate	Regurgitate	7	1	1.1
Iles Nuageuses	Cherel et al 2002	Congiopodidae	*Zanclorhynchus spinifer*	1994	Regurgitate	Regurgitate	8.6	2.7	0.8
Southern Brazil	Colabuono and Vooren 2007	Elasmobranchii	*Unidentified Elasmobranchii*	1994-2004	Whole stomach	Whole stomach	4	1	
Southern Brazil	Colabuono and Vooren 2007	Engraulidae	*Lycengraulis grossidens*	1994-2004	Whole stomach	Whole stomach	2	1	<1

Diego ramirez	Arata and Xavier 2003	Gadidae	*Micromesistius australis*	2001	Regurgitate	Regurgitate	4.7	0.4	2.6
Diego ramirez	Arata and Xavier 2003	Gadidae	*Micromesistius australis*	2002	Regurgitate	Regurgitate	4.6	0.5	3.7
New Island	Thompson 1992	Gadidae	*Micromesistius australis*	1987		Regurgitate			
Beaucheche Island	Thompson 1992	Gadidae	*Micromesistius australis*	1991		Regurgitate			
Falkland Islands	Thompson and Riddy 1995	Gadidae	*Micromesistius australis*	1991		Observation			
Kerguelen	Cherel et al 2000	Gempylidae	*Paradiplospinus gracilis*	1994, 1995	Regurgitate	Regurgitate	0.9	0.1	<0.1
South Georgia	Croxall et al 1999	Gempylidae	*Paradiplospinus gracilis*	1994	Regurgitate	Regurgitate	2.6	5.9*	0.8
Diego ramirez	Arata and Xavier 2003	Gonostomatidae	*Photichthys argenteus*	2002	Regurgitate	Regurgitate	1.1	0.1	0
Kerguelen	Cherel et al 2000	Harpagiferidae	*Harpagifer spinosus*	1994, 1995	Regurgitate	Regurgitate	3.5	0.5	<0.1
South Georgia	Prince 1980	Lamprey	*Geotria australis*	1976		Regurgitate			
Diego ramirez	Arata and Xavier 2003	Macrouridae	*Macrouridae* indet.	2001	Regurgitate	Regurgitate	1.6	0.1	
Kerguelen	Cherel et al 2000	Macrouridae	*Macrourus carinatus/holotrachys*	1994, 1995	Regurgitate	Regurgitate	2.6	0.4	3
South Georgia	Xavier et al 2003	Macrouridae	*Macrourus holotrachys*	2000	Regurgitate	Regurgitate	3	3	5
Diego ramirez	Arata and Xavier 2003	Macruronidae	*Macruronus magellanicus*	2000	Regurgitate	Regurgitate	26.9	3.6	88.9
Diego ramirez	Arata and Xavier 2003	Macruronidae	*Macruronus magellanicus*	2001	Regurgitate	Regurgitate	23.4	6.4	66
Diego ramirez	Arata and Xavier 2003	Macruronidae	*Macruronus magellanicus*	2002	Regurgitate	Regurgitate	36.8	9.1	75.8
Kerguelen	Cherel et al 2000	Melamphaidae	*Melamphaidae sp.*	1994, 1995	Regurgitate	Regurgitate	0.9	0.1	<0.1
Diego ramirez	Arata and Xavier 2003	Melanonidae	*Melanonus gracilis*	2001	Regurgitate	Regurgitate	1.6	0.1	
Diego ramirez	Arata and Xavier 2003	Melanonidae	*Melanonus gracilis*	2002	Regurgitate	Regurgitate	3.4	0.4	
Steeple Jason Island	Thompson 1992	Merlucciidae	*Merluccius* sp	1988		Regurgitate			
Falkland Islands	Thompson and Riddy 1995	Merlucciidae	*Macruronus magellanicus*	1991		Observation			
Falkland Islands	Thompson and Riddy 1995	Merlucciidae	*Merluccius* sp	1991		Observation			
Kerguelen	Cherel et al 2000	Moridae	*Laemonema kongi*	1994, 1995	Regurgitate	Regurgitate	0.9	0.1	<0.1
Falkland Islands	Thompson and Riddy 1995	Moridae	*Salilota australis*	1991		Observation			
Southern Brazil	Colabuono and Vooren 2007	Mugilidae	*Mugil* sp.	1994-2004	Whole stomach	Whole stomach	4	1	<1
Kerguelen	Cherel et al 2000	Muraenolepididae	*Muraenolepis marmoratus/orangiensis*	1994, 1995	Regurgitate	Regurgitate	12.3	1.8	3
Iles Nuageuses	Cherel et al 2002	Muraenolepididae	*Muraenolepis marmoratus/orangiensis*	1994	Regurgitate	Regurgitate	5.7	1.8	1.1
Diego ramirez	Arata and Xavier 2003	Myctophidae	*Electrona antarctica*	2002	Regurgitate	Regurgitate	2.3	0.5	0.1

Diego ramirez	Arata and Xavier 2003	Myctophidae	*Electrona carlsbergi*	2002	Regurgitate	Regurgitate	2.3	12.3	2.1
Diego ramirez	Arata and Xavier 2003	Myctophidae	*Gymnoscopelus hintonoides*	2002	Regurgitate	Regurgitate	1.1	0.3	0.2
Diego ramirez	Arata and Xavier 2003	Myctophidae	*Gymnoscopelus* sp.	2002	Regurgitate	Regurgitate	4.6	0.6	
Diego ramirez	Arata and Xavier 2003	Myctophidae	*Myctophidae indet.*	2002	Regurgitate	Regurgitate	1.1	0.1	
Kerguelen	Cherel et al 2000	Myctophidae	*Krefftichthys anderssoni*	1994, 1995	Regurgitate	Regurgitate	0.9	0.1	<0.1
Kerguelen	Cherel et al 2000	Myctophidae	*Protomyctophum bolini*	1994, 1995	Regurgitate	Regurgitate	0.9	0.1	<0.1
South Georgia	Croxall et al 1997	Myctophidae	*Gymnoscopelus bolini*	1986	Regurgitate	Regurgitate	2.3	5.3*	2
South Georgia	Croxall et al 1997	Myctophidae	*Krefftichthys anderssoni*	1986	Regurgitate	Regurgitate	2.3	5.3*	<0.1
South Georgia	Prince 1980	Myctophidae	*Myctophid* sp	1976		Regurgitate			
Kerguelen	Cherel et al 2000	Nototheniidae	*Dissostichus eleginoides*	1994, 1995	Regurgitate	Regurgitate	21.9	3.9	18.3
Kerguelen	Cherel et al 2000	Nototheniidae	*Gobionotothen acuta*	1994, 1995	Regurgitate	Regurgitate	1.8	0.5	<0.1
Kerguelen	Cherel et al 2000	Nototheniidae	*Lepidonotothen mizops*	1994, 1995	Regurgitate	Regurgitate	3.5	0.7	0.1
Kerguelen	Cherel et al 2000	Nototheniidae	*Lepidonotothen squamifrons*	1994, 1995	Regurgitate	Regurgitate	21.9	3.2	11.6
Kerguelen	Cherel et al 2000	Nototheniidae	*Notothenia cyanobrancha*	1994, 1995	Regurgitate	Regurgitate	14.9	2.3	4.5
Kerguelen	Cherel et al 2000	Nototheniidae	*Notothenia rossii*	1994, 1995	Regurgitate	Regurgitate	3.5	0.5	4.6
Kerguelen	Cherel et al 2000	Nototheniidae	*Paranotothenia magellanica*	1994, 1995	Regurgitate	Regurgitate	2.6	0.4	0.3
Kerguelen	Cherel et al 2000	Nototheniidae	*Unidentified Nototheniidae*	1994, 1995	Regurgitate	Regurgitate	4.4	0.6	2.2
Iles Nuageuses	Cherel et al 2002	Nototheniidae	*Dissostichus eleginoides*	1994	Regurgitate	Regurgitate	42.9	15.9	31.6
Iles Nuageuses	Cherel et al 2002	Nototheniidae	*Lepidonotothen squamifrons*	1994	Regurgitate	Regurgitate	2.9	0.9	1.4
Iles Nuageuses	Cherel et al 2002	Nototheniidae	*Notothenia rossii*	1994	Regurgitate	Regurgitate	2.9	0.9	3.2
Iles Nuageuses	Cherel et al 2002	Nototheniidae	*Paranotothenia magellanica*	1994	Regurgitate	Regurgitate	2.9	0.9	0.2
Iles Nuageuses	Cherel et al 2002	Nototheniidae	*Unidentified Nototheniidae*	1994	Regurgitate	Regurgitate	5.7	1.8	2.7
South Georgia	Croxall et al 1997	Nototheniidae	*Patagonotothen guntheri*	1986	Regurgitate	Regurgitate	13.9	78.9 *	14.9
South Georgia	Croxall et al 1999	Nototheniidae	*Nototheniidae* sp.	1994	Regurgitate	Regurgitate	2.6	29.4 *	0.1
Kerguelen	Cherel et al 2000	Paralepididae	*Magnisudis prionosa*	1994, 1995	Regurgitate	Regurgitate	0.9	0.1	0.1
South Georgia	Xavier et al 2003	Paralepididae	*Magnisudis prionosa*	1997	Regurgitate	Regurgitate	3	6	4
South Georgia	Xavier et al 2003	Paralepididae	*Magnisudis prionosa*	1999	Regurgitate	Regurgitate	13	7	7
South Georgia	Xavier et al 2003	Paralepididae	*Magnisudis prionosa*	2000	Regurgitate	Regurgitate	12	10	7
South Georgia	Croxall et al 1999	Paralepididae	*Magnisudis prionosa*	1994	Regurgitate	Regurgitate	10.5	23.5 *	22.1
South Georgia	Croxall et al 1997	Petromyzontidae	*Geotria australis*	1986	Regurgitate	Regurgitate	2.3	5.3*	0.9
Southern Brazil	Colabuono and Vooren 2007	Phycidae	*Urophycis brasiliensis*	1994-2004	Whole stomach	Whole stomach	2	1	2

Southern Brazil	Colabuono and Vooren 2007	Pomatomidae	*Pomatomus saltatrix*	1994-2004	Whole stomach	Whole stomach	7	3	7
Kerguelen	Cherel et al 2000	Rajidae	*Bathyraja* sp.	1994, 1995	Regurgitate	Regurgitate	14	2	4.5
Southern Brazil	Colabuono and Vooren 2007	Sciaenidae	*Cynoscion guatucupa*	1994-2004	Whole stomach	Whole stomach	2	2	3
Southern Brazil	Colabuono and Vooren 2007	Sciaenidae	*Macrodon ancylodon*	1994-2004	Whole stomach	Whole stomach	2	1	<1
Southern Brazil	Colabuono and Vooren 2007	Sciaenidae	*Micropogonias furnieri*	1994-2004	Whole stomach	Whole stomach	2	1	2
Southern Brazil	Colabuono and Vooren 2007	Sciaenidae	*Paralonchurus brasiliensis*	1994-2004	Whole stomach	Whole stomach	11	4	1
Southern Brazil	Colabuono and Vooren 2007	Sciaenidae	*Pogonias cromis*	1994-2004	Whole stomach	Whole stomach	2	1	<1
Southern Brazil	Colabuono and Vooren 2007	Sciaenidae	Unidentified Sciaenidae	1994-2004	Whole stomach	Whole stomach	4	3	2
Brazil	Petry et al 2007	Sciaenidae	*Paralonchurus brasiliensis*	1997-1998	Whole stomach	Whole stomach	5.7		
Brazil	Petry et al 2007	Sciaenidae	*Ctenosciaena gracilicirrhus*	1997-1998	Whole stomach	Whole stomach	2.9		
Diego ramirez	Arata and Xavier 2003	Sebastidae	*Sebastes oculatus*	2002	Regurgitate	Regurgitate	1.1	0.1	1.9
Southern Brazil	Colabuono and Vooren 2007	Trichiuridae	*Trichiurus lepturus*	1994-2004	Whole stomach	Whole stomach	2	1	2
Brazil	Petry et al 2007	Trichiuridae	*Trichiurus lepturus*	1997-1998	Whole stomach	Whole stomach	2.9		
Southern Brazil	Colabuono and Vooren 2007	Triglidae	*Prionotus punctatus*	1994-2004	Whole stomach	Whole stomach	4	2	1
Diego ramirez	Arata and Xavier 2003	unknown	unknown	2000		Regurgitate	7.7	0.2	
Kerguelen	Cherel et al 2000	unknown	unknown	1994, 1995	Regurgitate	Regurgitate	9.6	1.5	1.8

*% of n is for fish not overall

Appendix 5.2: Natural accessibility of main fish prey to BBA during the sampling period at each site. * indicate species that were unlikely to be obtained naturally unless scavenged as carrion.

Family	Taxa	Area	Sampling period	Accessible period	Fish stage	Reference
Clupeidae	*Sprattus fuegensis*	Falklands	October-March	All year	Larvae-Adult	(Ehrlich et al. 1999)
		Magellan Straits	February	All year	Larvae-Adult	A.Kusch (per obs)
Gadidae	*Micromesistius australis**	Falkland Islands	October-March	Winter/spring	Larvae	(Cohen et al. 1990, Ehrlich et al. 1999)
Macruronus	*Macruronus magellanicus**	Falkland Islands	October-March	Spring	Larvae	(Ehrlich et al. 1999)
Ophidiidae	*Genypterus blacodes*	Falkland Islands	October-March	All year	Adults	(Stevenson 2004, Roberts et al. 2015)
Bathydraconidae	*Parachaenichthys georgianus*	South Georgia	January-February	Spring/summer	Larvae	(Loeb et al. 1993)
Centrolophidae	*Icichthys australis*	South Georgia	January-February	All year	Larvae - Adult	(Roberts et al. 2015)
Channichthyidae	*Champsocephalus gunnari*	South Georgia	January-February	All year	Larvae - Adult	(Loeb et al. 1993, Miller 1993, Belchier and Lawson 2013)
	Pseudochaenichthys georgianus	South Georgia	January-February	All year	Larvae-Adult	(Iwami and Kock 1990, Loeb et al. 1993, Belchier and Lawson 2013)
	Chaenocephalus aceratus	South Georgia	January-February	All year	Larvae - Adult	(Loeb et al. 1993, Belchier and Lawson 2013, Cheung et al. 2013)
	Channichthys rhinoceratus	Kerguelen	November-January	All year	Larvae - Adult	(Loeb et al. 1993, Miller 1993)
Muraenolepididae	*Muraenolepis (microps/orangiensis)*	South Georgia	January-February	September-January	Larvae - Adult	(Loeb et al. 1993, Belchier and Lawson 2013, Cheung et al. 2013)
Nototheniidae	*Gobionotothen sp.*	South Georgia	January-February	October-March	Larvae	(Belchier and Lawson 2013)
	Notothenia rossii	South Georgia	January-February	Spring/summer	Larvae/ juvenile	(Loeb et al. 1993)
	Lepidonotothen larseni	South Georgia	January-February	Spring/summer (larvae) all year adults	Larvae - Adult	(DeWitt et al. 1990, Loeb et al. 1993)
	*Dissostichus eleginoides**	Kerguelen	November-January	Winter/Spring	Larvae	(Loeb et al. 1993, Mori et al. 2016)
	Lepidonotothen squamifrons	Kerguelen	November-January	Summer/Autumn	Larvae	(Loeb et al. 1993, Koubbi et al. 2000)
	Paranotothenia magellanica	Macquarie Island	November-March	All year	Larvae	(Kock and Kellermann 1991, Miller 1993)
	*Patagonotothen (ramsayi)**	Falkland Islands	October-March	Only dead individuals	Adults	(Nakamura et al. 1986)

	Patagonotothen tessellata/brevicauda	Magellan Straits	February			
	Patagonotothen guntheri	South Georgia	January-February	All year	Adults	(DeWitt et al. 1990)
Rajidae	*Bathyraja**	Kerguelen	November-January	Only dead individuals		
Congiopodidae	*Zanclorhynchus spinifer*	Macquarie Island	November-March	All year	Larvae, possibly adults in the shallows	(Heemstra and Duhamel 1990, Loeb et al. 1993)
Bramidae	*Unidentified sp.*	Macquarie Island	November-March	All year	Larvae - Adult	(Roberts et al. 2015)
Myctophidae	*Lampanyctus (intricarius/articus)*	Macquarie Island	November-March	All year	Adult	(Hulley 1990)

Chapter 6 - General discussion and future directions

"Now is no time to think of what you do not have.
Think of what you can do with that there is"

Ernest Hemingway
The Old Man and the Sea

6.1 Overview

Dietary studies provide an important tool for evaluating how changing environmental conditions and commercial fishing may influence seabird populations (Cherel and Klages 1998, Croxall et al. 1999, Pinaud et al. 2005, Chiaradia et al. 2010). Using albatross as a model, this thesis has highlighted key gaps in the spatial and temporal dietary information collected, which impedes our ability to identify and monitor these threats. Using DNA metabarcoding to assess the overall and fish-specific components of albatross diets, this thesis has demonstrated that DNA metabarcoding provides an alternate or complementary diet analysis method for seabird populations. DNA metabarcoding has significant advantages over current alternatives and can increase our knowledge of the ecology of seabirds and their prey, including gelatinous organisms, the level of interaction with fisheries, and an ongoing mechanism to assess environmental changes through ecosystem monitoring.

This final thesis chapter provides an overview of new insights we have gained into albatross diets and the use of dietary DNA metabarcoding for seabird populations, discussion on some of the technical considerations and challenges when using these methods, and future applications for DNA metabarcoding of scats from seabirds and land-based marine predators.

6.2 New insights into albatross dietary studies and prey

This thesis has highlighted that the amount of albatross prey data identified to high taxonomic resolution has declined over time (Chapter 2). There are also few breeding sites for which long-term dietary datasets exist. Long-term monitoring programs are difficult to maintain as they can be costly and difficult to fund (Lindenmayer and Likens 2010) and the invasive nature of conventional dietary techniques can limit their use (Delord et al. 2011, DSEWPAC 2011). However, this thesis has showcased the value of molecular diet analysis methods to help fill some of these knowledge gaps and increase the number of sites with high taxonomic resolution diet data. This included two black-browed albatross colonies for which no previous diet data existed (Albatross Islet and Macquarie Island). Both of these populations had a high occurrence of fish species in their diet that had not previously been detected for this species (sprat and Antarctic horsefish).

The circumpolar examination of black-browed albatross diet has shown that there is significant variation in the diet of a single species across its breeding range. Although fish was the main prey species consumed, the contribution of fish to the diet varied between sites, with lower trophic prey such as jellyfish and crustaceans regularly occurring at some sites. The level of fishery discards in the

diet also varied considerably between colonies, including between breeding colonies in relatively close proximity. Interestingly, however, there was little inter-annual variability in either the overall diet or the fish species consumed, which suggests that the prey availability was fairly consistent in the years studied. Consequently, gelatinous prey or fisheries discards in the diet are not likely to be isolated incidents, but may be regular feeding behaviours at these sites. The combination of broad dietary data and fish-specific data has shown that threats posed by both commercial fishery interactions and climate change will likely vary considerably between breeding populations. Previous studies examining multiple colonies using stable isotopes also found variability in the trophic level of black-browed albatross (Cherel et al. 2013, Jaeger et al. 2013). The current study provides another level of detail to this work to explore which prey groups contribute to these changes. Long-term diet monitoring across multiple breeding colonies and breeding stages will be valuable to monitor how shifts in prey availability from changing climate and fishing practices may affect the breeding success and survival of albatross and if their plasticity to adapt to these changes varies globally.

6.2 Application of DNA metabarcoding to assess seabird diets

DNA metabarcoding of scats provides an alternative dietary method for identifying, evaluating and monitoring the diet of seabird populations (Chapter 4 and 5). The optimised scat collection protocols developed in this thesis allow dietary data to be collected during all breeding stages and from all age cohorts. High quality dietary data can be obtained by: collecting fresh scats; minimising contamination from dirt and vegetation; and targeting birds recently returned to the colony. Although the amount of non-target DNA is higher in samples from small chicks and fasting adults, careful collection of larger sample sets still allow dietary information to be obtained from these birds. This is one of the advantages of DNA metabarcoding compared to other dietary analysis methods, as it can be applied during all breeding stages. Collection of dietary data from adults during the incubation and brood stages is especially valuable, as stomach content samples are usually only available from chicks during chick rearing (Chapter 2).

The circumpolar study of black-browed albatross diet in this thesis demonstrated the value of DNA metabarcoding dietary methods for ecosystem monitoring programs and assessing interactions between albatross and commercial fisheries. One of the most important components of an ecosystem monitoring program is the ability to detect changes in the marine ecosystem (Constable 2002). If those changes involve a group of animals that cannot be detected by current monitoring programs, then it throws into question the efficacy of such programs. The high detection rate of gelatinous prey in the diet of black-browed albatross presented here highlights a major limitation of

studies that rely on stomach contents analysis to identify the prey of apex predators. Ecosystem monitoring programs that use stomach contents may be unable to detect what could be a major prey group of these chosen indicator species. The use of universal primers has highlighted the high occurrence of scyphozoan jellyfish in albatross diets (Chapter 4) and in Adélie penguin diets (Jarman et al. 2013, McInnes et al. 2016a). Increases in gelatinous prey abundance have occurred in many oceans (Brodeur et al. 2002, Lynam et al. 2006) and are predicted to intensify under current climate change scenarios (Purcell 2005, Quiñones et al. 2015). Future seabird diet monitoring programs should be designed to detect changes across the full prey spectrum, including jellyfish, so that changes to food-webs and ecosystems can be properly evaluated.

DNA metabarcoding is a useful dietary tool for assessing fish prey diversity (Chapter 5, Bowser et al. 2013). In chapter 5, interactions between albatross and commercial fisheries were investigated across a circumpolar range. DNA metabarcoding enabled a higher diversity of fish prey to be identified than stomach contents analyses that rely solely on otoliths (Reid et al. 1996, Xavier et al. 2003a) and a similar diversity of species to studies using multiple body parts (Cherel et al. 2000b). However, DNA methods can be applied to multiple breeding stages, which enabled high incidence of discard consumption to be identified during incubation at the Falkland Islands. This method could also be applied to sites such as Macquarie Island, where conventional dietary methods are considered too invasive (DSEWPAC 2011). DNA metabarcoding has enabled the detection of fishery discards in the diets of multiple albatross populations, identifying an elevated level of risk of incidental albatross mortality. DNA metabarcoding provides a mechanism for fishery management authorities to identify fish prey diversity, monitor the implementation and adherence of vessels to mitigation measures, and evaluate the effects of discard policies on interactions between seabirds and vessels. The reduction in fishery discards is of key importance to reduce the risk of incidental mortality of seabirds (Abraham et al. 2009).

6.3 Technical challenges and considerations with DNA metabarcoding

Although there are many advantages to using DNA metabarcoding in dietary assessment, there are a number of technical difficulties and drawbacks that should be considered when planning vertebrate dietary studies. These include taking into account the limitations of the data obtained (e.g. DNA metabarcoding analysis cannot provide estimates of consumed prey body size) as well as project planning considerations such as sample collection method, and technical molecular challenges. These limitations and considerations are outlined below.

6.3.1 No prey size estimates

One issue with using DNA metabarcoding for assessing diet is the inability to determine the meal size, prey size or prey parts consumed, which is also a limitation of any biochemical analyses such as stable isotope analysis. Stomach content studies enable an approximate estimate of these metrics using the size of hard parts such as otoliths and squid beaks (Barrett et al. 2007). This is particularly useful for estimating the level of interaction with fisheries as the prey size and body parts can provide a proxy for where the prey may have originated (Thompson 1992). In chapter 5, I relied on the depth profile of the fish in conjunction with fishery catch data to determine which prey likely originated from fisheries as well as potential competition between albatross and fisheries. However, there may be prey items that are naturally accessible to albatross that are also obtained through fishery discards that would have been excluded using these methods, underestimating the interaction with the fishery. Additionally, any potential competition for fish species between albatross and fisheries is based purely on species presence rather than a size class of fish. If the fishery and the albatross are targeting fish of different life stages, then any potential competition may be overestimated. Collection of some regurgitates or opportunistic prey collections from around the colony would enable the parts of the fish consumed to be assessed and the size/age class of fish to be established (Granadeiro and Silva 2000). An integrated approach for diet monitoring of albatross populations was proposed in Chapter 2, including how data could be collected (Table 2.5).

6.3.2 Project planning and sample collection

The optimised scat collection protocols developed during this study highlight the value of robust experimental design and planning before commencing scat collection. Identifying the research question and what samples are required to answer it is imperative to ensure high quality data is collected (King et al. 2008). However, given the difficult field conditions ideal collection scenarios are not always possible. For example even though fresh scat samples were targeted during the black-browed albatross study, these were not always obtained. This may have been due to local conditions, experience of the collector, or time available for the task. Although collection of scats seems like an easy task, it does require some training with detailed instructions on how and what to collect. To ensure high quality samples are collected in future studies, the information in Chapter 3 and the supplementary video guide aim to provide sufficient information for non-experienced personnel to obtain high quality samples. These protocols will be incorporated into the 'Guidelines for Seabird Dietary Studies' by The Agreement for the Conservation of Albatross and Petrels and will be translated into several languages to assist researchers globally (Appendix 6.1).

6.3.3 Marker choice

One challenge of working with scats is that the DNA is highly degraded, therefore only short fragments can be amplified (King et al. 2008). I chose the 16S gene region for fish identification in this study as it is well conserved for PCR primer binding when compared to the commonly used cytochrome c oxidase (COI) gene region. It also has greater species identification potential than the 18S gene (Deagle et al. 2014). In this project, the 16S primer set was designed to amplify as many Southern Ocean fishes as possible, identified using GenBank. Four of the fish genera in chapter 5 (*Patogonotothen, Muraenolepis, Merluccius* and *Champsocephalus*) contained multiple species that were genetically similar within the target gene region. To identify the species in these genera, alternative PCR primer sets would be needed. In the case of *Merluccius* and *Champsocephalus*, there are minimal sequence differences across the entire 16S region, which makes this difficult.

For the case study in chapter 5, I only explored the fish component of the diet and fishery overlaps with target and by-catch fish species, because a cephalopod fishery only occurred at the Falkland Islands. A cephalopod and fish primer multiplex could be used in future studies to cover both prey groups and would be a useful addition to studies in these regions. Using the two primer sets, frequency of occurrence could be compared; however, the relative read abundance of prey items cannot be compared. This is where the use of the universal metazoan primer (18S_SSU) is valuable in the first instance to screen all samples to assess the overall contribution of fish and other prey items.

6.3.4 Species assignment

Studies that require exact species identification of dietary items are generally limited by the quality of DNA databases for identifying metabarcode sequences. Ideally, before commencing a DNA metabarcoding study using group specific markers, a sample from each food item should be obtained and sequenced to make a local genetic library (Deagle et al. 2009, Lopes et al. 2015). However, for studies over a broad geographic range, or where there is no *a priori* knowledge of diet, this may be impractical (Emami-Khoyi et al. 2016). To identify the fish prey of black-browed albatross, I was able to obtain tissue samples for most of the main fish prey species missing from GenBank. However, a few species remained unrepresented in the database, which I could not assign with confidence.

Automated software that assigns taxonomic classification from GenBank data can be an efficient way to assign sequence reads. However, there are two potential errors generated when using automated software to assign species matches: 1) when there is no close match on GenBank and the

hits are not genetically similar to the reference sequence; or 2) if there are species missing from the database the sequence may be incorrectly assigned to a genetically similar species within the same taxonomic group. There are several metrics that can be used to improve the first error such as comparing E values and query cover. Software such as MEGAN achieves this using the Lowest Common Ancestor algorithm, which allows the conservation level of the reference sequence to be compared to sequences on the database and assigns classification to the highest taxonomic level available (Huson et al. 2007). However, the second error is still problematic using this software as it relies on a complete database. GenBank is a general purpose DNA sequence database that is not specifically designed for DNA species identification. It does not contain all sequences for a taxonomic group like ray-finned fishes (class Actinopterygii). For example, a sequence amplified by my 16S fish primers was assigned by MEGAN to the duckbilled barracudina (*Magnisudis atlantica*); however, there was no sequence available for the southern barracudina (*M. prionosa*) which is a more likely assignment. If both were present in GenBank, then the lowest taxonomic level MEGAN would assign is genus. For the fish primer set, I relied on MEGAN for an initial filter of the data and then manually checked each species and genus on GenBank, which was time consuming, but allowed greater confidence in the results. It is unclear in many studies how missing species data is accounted for, especially in studies where only the top BLAST matches are used (Emami-Khoyi et al. 2016). A conservative taxonomic approach to identification and clear stipulation of which species could not be confirmed would be useful for future studies.

6.3.5 Comparison metrics

Dietary studies using stomach contents typically quantify diet using multiple metrics. Each metric has inherent biases and limitations, but when used together, they provide a greater insight into the importance of items in the diet and allow a more critical assessment of food consumed (Duffy and Jackson 1986, Barrett et al. 2007). Frequency of occurrence (FOO) alone may lead to overestimation of abundance of a common prey item that is eaten in very small amounts, whereas the average mass of a prey item can appear more common if eaten in large amounts by few animals. For example, in the BBA diet at South Georgia in 1994, krill constituted 24% FOO, but only 5% mass, compared to South Georgian Icefish that were 11% FOO, but 41% by mass (Croxall et al. 1999). If either one of these metrics is reported alone, two alternate views of the diet are shown, whereas in combination they provide a better estimate of the true diet.

For DNA metabarcoding-based dietary data, the commonly-used metric to report data is FOO, which allows a simple comparison of which prey items are present (Bowser et al. 2013). However, it can

inflate the importance of food items that represent only a small fraction of the diet, including secondary prey items. The average proportion of sequences, or relative read abundance (RRA), is another metric used to present dietary sequence data (Willerslev et al. 2014, Kartzinel et al. 2015). This method has been validated using stable isotope data (Kartzinel et al. 2015) and feeding trials (Deagle et al. 2010, Willerslev et al. 2014). Again, this method is not free of biases and the proportion of food consumed may not exactly match the number of sequences obtained; however, the RRA of prey items is consistent (Deagle et al. 2010). Throughout this thesis I have presented both FOO and the proportion of sequences to give a broader view of the diet than only one metric allows. The only exception was Chapter 3, where those experiments were specifically testing the relative proportion of sequences obtained under different conditions. Presentation of both metrics provides a broader picture than a single metric.

For long-term monitoring programs, standardised reporting of DNA dietary data and methodology would be valuable to ensure consistency, reproducibility and replication of diet studies. Yilmaz et al. (2011) proposed the 'minimum information about a marker gene sequence' (MIMARKS) that should be collected for sequence data. This includes the basic spatial and temporal information that most studies report, but also specific information about the target gene, sequencing method and environmental package (e.g. scats). In addition to these parameters, information on the prey group (or species), sequence counts, relative read abundance and FOO would also be beneficial. A proposed template for the collection and maintenance of dietary data is provided in Appendix 6.2, and would be a valuable resource for dietary databases such the one established for the Southern Ocean (Raymond et al. 2011).

6.3.6 Sequencing depth

The sequencing depth can affect the diversity of DNA within a sample through false negatives and false positives (Alberdi et al. 2017, DiBattista et al. 2017, Lanzén et al. 2017). However, knowing the optimal sequencing depth to minimise each of these is difficult. Low sequence depth will increase the risk of false negatives, whereas a higher sequencing depth could increase the risk of false positives through amplification of contamination. Throughout this research, I used 100 sequence reads of food DNA as a cut-off for a sample to be included in the study. This was to ensure that any samples with low sequence reads would not create significant biases in either RRA or FOO analyses.

The occurrence of false negatives was unlikely to be a major concern for this study as I was examining the diet at a population-level rather than for individuals and in most cases I had relatively

high sample sizes which meant that the majority of important prey items should have been detected. However, there may have been a number of false positives that were inadvertently included in the data produced. The 100 read cut-off was based on my negative controls containing either no DNA reads or very low sequence reads. Although the relative read abundance is unlikely to vary with an increase in sequencing depth, the frequency of occurrence results could change considerably as FOO calculations were based on food items which comprised >1% of food sequences for that sample. Therefore, if the total number of food DNA reads was close to the cut-off, then only one or two sequences were needed for that species to be included. Low-level contamination by a few sequences is likely not just during PCR amplification but also in the field during sample collections. The use of both RRA and FOO enabled the identification of food items that were high in occurrence, but actually represented a low proportion of reads. This discrepancy was particularly noticeable when sample sizes were low, such as for the samples from Diego Ramirez. Further work is needed to test the effect of sequencing depth on diet results and the effect of varying cut-offs for sample inclusion overall and for FOO calculations. These analyses would be especially valuable when interpreting datasets with low sample sizes or where the contamination risk is high.

6.3.7 Prey detection

The 18S primer set designed for this study were tested by aligning sequences from all major prey groups and tested with both flesh DNA and scat samples. The target region was of similar length in all major prey groups which meant that the chance of amplification bias was likely to be low. However, there were some prey groups that were detected in the diet less commonly than expected from previous conventional diet studies. The prevalence of cephalopods in albatross diet was found to be relatively low compared to previous diet estimates using stomach contents analysis, which is discussed in detail in Chapter 4. There was no doubt that the universal primers are able to detect cephalopods. The primers are an exact match to cephalopod DNA, the sequences are similar length to other prey groups, and they amplified DNA from squid flesh and from scats from other species known to have consumed squid. It is possible that cephalopod DNA is more degraded after digestion than other species; however, previous feeding trials have not found this to be true, with cephalopods and fish prey both amplifying well (Casper et al. 2007). Although there have been studies comparing the proportion of food fed to captive animals with the DNA sequence obtained in scats, these have been done using group-specific primers rather than universal primers (Casper et al. 2007, Deagle et al. 2010). Therefore, biases in DNA amplification towards a particular prey group are possible; however, I believe in this study the impact of this is likely to be low. In chapter 4, the RAA and FOO were both used and the latter analyses will detect cephalopod ingestion even if there is a

bias against amplification of their DNA relative to other prey groups. The overall conclusions of these analyses are similar: that cephalopod occurrence was low. However, further work would be valuable to test whether there are any biological factors (e.g. digestion rate and meal size) or technical factors (e.g. primer match, G-C content, DNA quality) that may cause detection biases for different phyla.

It could not be determined during this study whether the low occurrence of cephalopod DNA was due to a molecular bias, low prey availability, sampling timing or previous overestimation of prevalence in the diet using conventional methods. DNA metabarcoding provides a tool for comparing the relative proportion of prey sequences between sample sets, but cannot easily provide absolute data on the prey consumed (Deagle et al. 2013). However, this is also the case for stomach contents studies where differential digestion can affect the data obtained. A comparative study with scats and stomach contents collections could provide some answers; however, interpretation of the results from these comparisons is difficult. The scats contain DNA from digested food, whereas stomach contents contain remains from undigested food. Therefore even a direct comparison of methods is comparing two different things. Feeding trials might present a means of conducting an unbiased experiment and would involve a group of birds fed a set diet, with scats collected from a proportion of birds and regurgitates obtained from another. While this would be highly invasive, it would directly highlight the inherent biases of all dietary methods compared to prey consumed.

6.4 Future applications

6.4.1 Effects of jellyfish consumption on seabird breeding success

The recent discovery of jellyfish in the diets of seabirds has highlighted a substantial gap in our understanding of marine food-webs (Chapter 4; Jarman et al. 2013, McInnes et al. 2016a, Thiebot et al. 2016). However, it is difficult to determine whether the recent observations of jellyfish in seabird diets are due to an increase in jellyfish availability, or increased jellyfish detection capability due to the use of new dietary methods. At the Falkland Islands, jellyfish were consumed by black-browed albatross in years of both low and high jellyfish abundance, suggesting they were actively selecting jellyfish rather than only consuming them during periods of high jellyfish availability (Chapter 4). Gelatinous prey are low in nutritional value as measured by calorimetry (Doyle et al. 2007); however, this may be offset by the relative ease of catching them, which would reduce the overall cost of foraging and potentially compensate for their low energy content. The use of DNA dietary methods in conjunction with information on meal mass (from chick mass), foraging trip duration and breeding success would allow us to investigate the role that jellyfish play in seabird diets. From this

assessment, predictions could be made about what effects an increase in jellyfish prevalence may have on seabird populations. This would likely require an increase in genetic information for jellyfish species, as there are gaps in the jellyfish data currently available on GenBank.

6.4.2 Parasite occurrence

DNA metabarcoding of scats can be applied to the study of parasites. Cestodes are the main endoparasites in pelagic seabirds and their presence is largely driven by diet, with fish, cephalopods and crustaceans likely intermediate hosts (Hoberg 1996). During the egg incubation period for both shy and black-browed albatross, samples had a high frequency of parasite occurrence. When this was examined more closely in the shy albatross samples, the occurrence of parasite DNA and relative sequence proportion increased with fasting duration of the bird. It is possible that parasites are more prevalent in certain prey during the incubation period, or that parasite eggs are laid during this time, thus increasing the amount of parasite DNA in the bird scat. In waved albatross (*Phoebastria irrorata*) parasite prevalence was greater in chicks than adults (Jiménez-Uzcátegui et al. 2015) and thought to be a result of reduced immunity. Those samples were collected at different times for adults and chicks (May and September respectively), therefore it is possible that the result may also be a reflection of a seasonal timing in parasite prevalence. However, if this were the case in our study, higher proportions would also be expected in all birds, not just those fasting for longer. Further research is needed to understand the relationship between fasting and endoparasite presence, diversity of endoparasites across albatross populations, and if parasite DNA presence in scats can be used to assess the relative health of a population.

6.4.3 Integrated network of predator diets and prey diversity

There is extensive scope to expand the application of dietary DNA metabarcoding. Future studies could explore diet from a single species to multiple predators, from island ecosystems to oceanic basins, and from top predators to their prey and parasites. DNA metabarcoding of scats from marine animals that return to land can provide the foundation for an integrated network of predator diets and prey diversity across broad geographic scales (Figure 6.1).

This thesis, in conjunction with other published studies (see below), has shown that DNA dietary methods can be applied to a wider range of individual groups within a species than conventional methods allow. This includes identifying diet differences between different breeding stages (Chapter 3, Jarman et al. 2013), developmental stages (Chapter 3, Bowser et al. 2013, McInnes et al. 2016a), and for different sexes which can be identified through DNA in the scats (Faux et al. 2014). This diet

information can provide insights into which age or sex classes of the population are likely to be at increased risk from fishery interactions and changing environmental conditions. These comparisons can then be applied to the species across multiple sites to test the site specificity of these dietary observations. This would be similar to work carried out in chapters 4 and 5 and on other species (Deagle et al. 2009, Jarman et al. 2013), but with greater depth of information within a site.

These detailed species-specific studies could also be expanded to incorporate multiple species within an ecosystem, which is more easily achieved with DNA based methods (Kartzinel et al. 2015). These multi-species studies may enable the question of prey availability to be assessed. As discussed in Chapter 4, it is often difficult to determine the availability of prey and whether predation is based on choice or availability. These multi-species studies can reveal which species are available and the importance of different prey groups at an ecosystem level (Moreno et al. 2016), including the importance of fishery species (Croxall et al. 1997). From this information, multiple top predator food-webs can be constructed and spatial foraging overlaps with fisheries from multiple species could be studied simultaneously, which would improve models for fishery ecosystem risk assessments (Constable et al. 2000).

Long-term dietary datasets for albatross have only been obtained for a small number of breeding sites (Chapter 2). The implementation and continuation of studies at the key dietary monitoring sites proposed in Chapter 2 would provide a foundation for prey changes to be assessed, with scope to expand to multiple species across sites. An integrated network of diet monitoring across multiple species and sites would improve our ability to track small and large-scale dietary shifts. At the time of writing, the Scientific Committee of Antarctic Research (SCAR) is expanding the Southern Ocean dietary database (Raymond et al. 2011). This new 'Diet and Energetics' database will contain published dietary data for top predators in the Southern Ocean, including fishes, seabirds and marine mammals. The albatross diet database developed in Chapter 2 has been incorporated into this database. This database provides the foundation to track prey distribution and prey prevalence across broad spatial and temporal scales. From this database, an assessment of the dietary knowledge gaps would highlight which islands or areas are data deficient. Scat collections at these sites combined with DNA metabarcoding could improve our knowledge of prey diversity in the area.

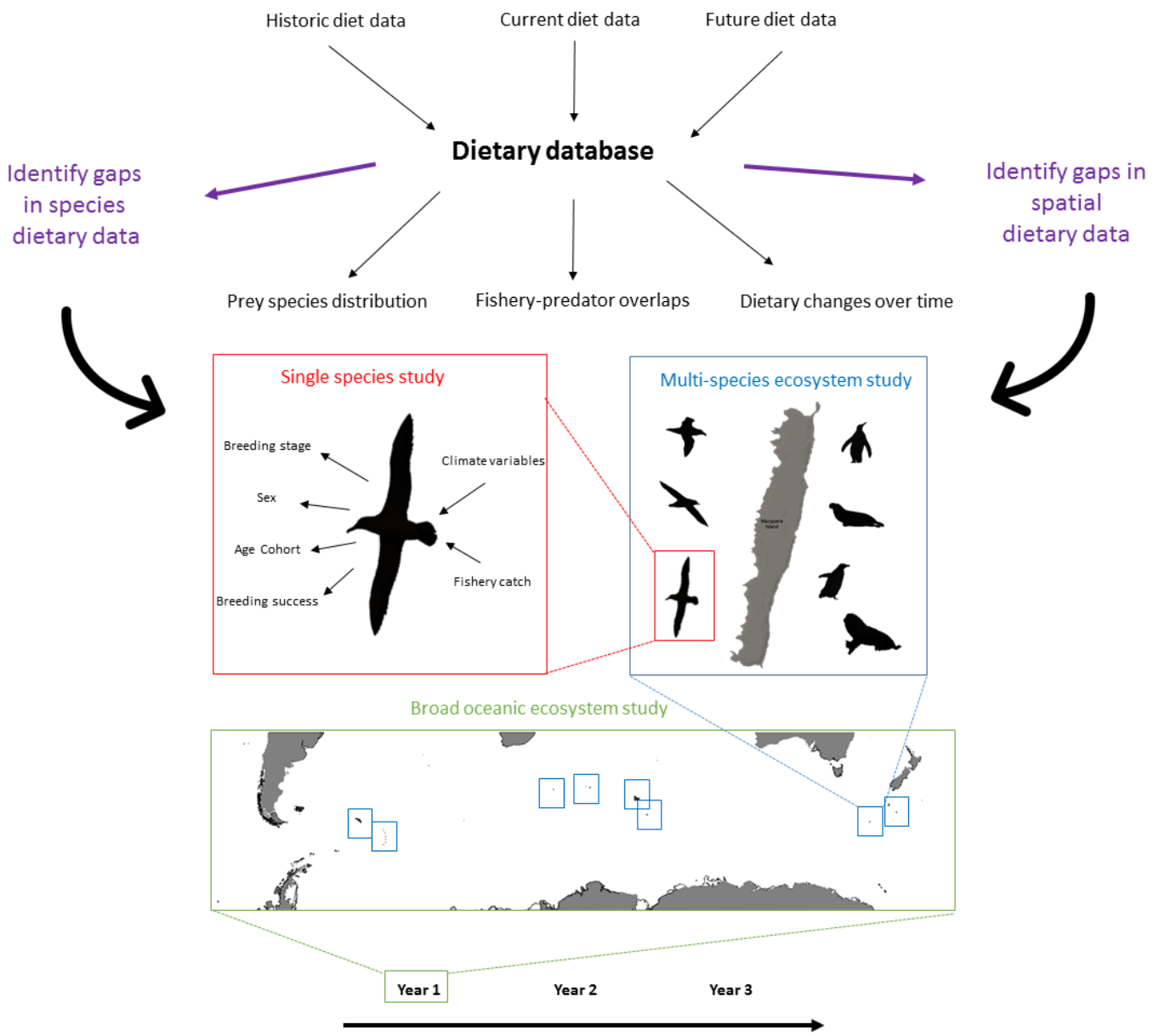

Figure 6.1 An integrated network of predator diets and prey diversity. This figure provides the key ideas for an expansive network of dietary information to increase our knowledge of marine ecosystems, from single species at one site to multiple species across broad oceanic ecosystems.

6.5 Closing remarks

The research conducted for this thesis has demonstrated that DNA metabarcoding of scats provides a valuable, non-invasive technique for assessing diet in albatross populations. The ability to obtain both broad dietary information and to identify prey groups with high taxonomic resolution, enables continued diet information to be collected over the long-term. The collection of samples in the field

requires care to ensure that high quality dietary data is obtained. However, using the field protocols developed in this thesis, dietary samples can be collected during all stages of the breeding season from chicks, breeders and non-breeders, which has not been possible previously using more conventional methods. DNA metabarcoding is especially useful for monitoring and evaluating the impact of fisheries interactions and environmental changes on albatross populations, including the detection of gelatinous prey, and allows long-term robust dietary data sets to be collected.

6.6 Appendices

Appendix 6.1 Field collection protocols for DNA dietary analysis of seabird scats to be incorporated into the ACAP 'Guidelines for Seabird Dietary Studies'

Background

Food DNA present in scats provides a non-invasive tool for studying the diet of seabirds (e.g.Deagle et al. 2007, Bowser et al. 2013, Jarman et al. 2013, McInnes et al. 2016a). Dietary DNA metabarcoding uses high-throughput sequencing of small, highly variable DNA regions that survive digestion to identify the food consumed (Pompanon et al. 2012). This may involve identification of a particular food species using species specific markers (Jarman and Wilson 2004); food within a taxonomic group using group specific markers (Jarman et al. 2004, Murray et al. 2011, Zeale et al. 2011); identification of all food taxa using universal metazoan markers (O'Rorke et al. 2012a, Jarman et al. 2013); or a combination of these approaches (Deagle et al. 2009, Bowser et al. 2013). However, characterising the entire diet requires 'universal' markers that are capable of amplifying DNA from any food group (King et al. 2008, Jarman et al. 2013).

Universal metazoan polymerase chain reaction (PCR) primers amplify from all eukaryotic DNA, but will inevitably also amplify unwanted DNA from non-food items (Deagle et al. 2009, O'Rorke et al. 2012a). Non-target DNA within the scat may originate from the animal being sampled, its parasites, gut flora; or contamination from external organisms such as insects and vegetation. These sources of DNA can overwhelm the sequences amplified from a sample, making detection of DNA from food items less effective. Sample sizes must consequently be increased to address the underlying questions of a study, increasing processing costs. In some cases, non-target DNA amplification can be reduced by using a blocking primer to suppress amplification of specific DNA types, such as DNA of the defecating animal (O'Rorke et al. 2012a). However, development of blocking primers is challenging and food sequences may be inadvertently blocked with this approach. The use of blocking primers becomes more complex when there are multiple non-target DNA groups present. To improve the quality and quantity of dietary information obtained from scat samples, these optimised scat collection protocols were developed using Shy Albatross (*Thalassarche cauta*) to provide a basis for future experimental designs, enable the collection of high quality diet samples and reduce non-target DNA amplification. Further details can be found in McInnes et al. (2016c).

Considerations prior to sampling

Careful planning of DNA dietary metabarcoding studies prior to sample collection is imperative for overall project success. Researchers should consider the dietary question they are targeting and focus on which scat samples will inform this. This includes marker selection, seasonal changes, fasting and the age of animals.

The ideal marker to choose will be based on the scientific question. Group specific markers can provide high taxonomic resolution of prey within a group (e.g. cephalopods), however will only detect that group of prey (Deagle et al. 2009, Bowser et al. 2013). A universal metazoan primer set will allow the all main prey groups to be identified, but with low taxonomic resolution (Deagle et al. 2009, Jarman et al. 2013, McInnes et al. 2016a). If a universal metazoan marker is chosen, additional care will be needed in the field to reduce the collection of non-food DNA.

DNA dietary analysis is an excellent tool for identifying diet at a population level, however identifying the diet of individuals using this method should be treated with caution. The digestion time is often unknown and depending of foraging duration, the scat may not relate directly to the previous meal.

Factors affecting sample success

Sample freshness

Fresh samples where the bird is observed defecating provide significantly more food DNA than dry samples (McInnes et al. 2017a). Dry scats have had more potential exposure to external contamination, particularly from fungi and also DNA within the scat is likely to degrade with UV exposure (Oehm et al. 2011). Recent scats that were wet, but the time since defecation was unknown had mixed success. Recent samples had a lower amplification success than fresh samples, but the proportion of food DNA detected in those that did amplify, was not significantly lower than that of fresh scats. Therefore using scats that are still wet may produce dietary information, but larger sample sizes would be required and reliance on small amounts of DNA may reduce data quality (Murray et al. 2015).

If the study uses group specific dietary markers, then food DNA may be detected for longer. For example, in carrion crows (*Corvus corone*), food DNA could be detected for up to five days when protected from UV and rain exposure (68% success), however, this was dramatically reduced when scats were left in exposed areas (17.5% success) (Oehm et al. 2011). Similarly, Steller sea lion (*Eumetopias jubatus*) scats also produced detectable prey DNA for up to five days in some samples using group specific markers (Deagle et al. 2005).

Collection substrate

Seabird colonies are often surrounded by tussock grass and exposed dirt, both of these provide contamination from other DNA sources through vegetation or unicellular organisms in the dirt. Consequently, the substrate the sample lands on can affect the amount of food DNA obtained. Scats that land on substrates that contain other sources of DNA, such as plant or dirt, will increase the risk of contamination (McInnes et al. 2017a). Samples collected from rock or ice enable more food DNA to be detected. If samples are obtained from dirt or vegetation, ensure that the collection of other soil or plant is minimised. Take care in colonies with multiple bird species, especially when they might be a possible prey item. For example if the target animal is known to feed on carrion and breeds around penguins or seals, ensure the DNA obtained is not contamination.

Breeding stage

The detection of food DNA in scat samples throughout the season is strongly linked to fasting (McInnes et al. 2017a). Longer periods of fasting during incubation cause a low detection of food DNA in scats, whereas food DNA detection can be much higher for breeding birds during brood. This is likely to be linked to more frequent and shorter feeding trips during this stage. During periods of fasting, non-target DNA was dominated by endoparasites and avian DNA. Depending on the aim of the study, try to collect samples from birds with the minimum time since feeding. During incubation, target birds that are back at the colony for less than a day. This may involve observing birds newly returned to the colony or marking one bird on the nest to monitor incubation length and know when they change over. Samples from non-breeding birds in the colony during brood also had a lower detection of food DNA, which was attributed to increased time at the colony and fasting.

Developmental stage

Collections from young animals are likely to pose problems for DNA dietary analysis with reduced detection of food in scats (McInnes et al. 2017a). As food is delivered by regurgitation, food items are likely to be partially digested before they are fed to the chick. Consequently, digestive processes may excessively degrade food DNA in chick scat samples. Additionally, there is presumably cross-over of parental DNA to the chick during regurgitation, which may cause the amount of bird DNA to be inflated, thereby reducing the food DNA proportionately. If a blocking primer is used that

supresses bird DNA, the amount of food DNA detected in chick scats can be increased, but care should be taken that the blocking primer doesn't block other vertebrates such as fish.
Scat samples from older chicks enabled a higher detection rate of food DNA than small chicks (McInnes et al. 2017a), which may reflect larger meals or a reduction in stomach oil. This oily liquid can contribute up to 80% of the sample mass in some albatross stomachs (Thompson 1992). In shy albatross, there is a greater mass of oily liquid in younger chicks than older chicks (Hedd & Gales 2001), which may dilute the food DNA.

Summary

DNA metabarcoding provides a valuable dietary tool to identify the prey in predator scats. This method can provide broad diet information across multiple taxon or species specific resolution of targeted prey groups. There are some benefits over hard-part analysis with the detection of soft-bodied prey and hard-bodied prey items that are usually retained in stomachs aren't overestimated. Samples can be collected during all breeding and life-history stages when scats are accessible (Bowser et al. 2013) however care should be taken during some breeding stages and ages as discussed in this document. As no handling of birds is required, it provides an ideal method of assessing the diet of sensitive species. The limitations are that prey age, size class and mass cannot be assessed. Scats must also be available, so the approach is not feasible for determining diet during the non-breeding period for species that remain far from land. The method is not currently commercially available (2016), however there are numerous research institutes that are using this method and as demand increases it will hopefully become commercially available in the future.

Collection methods

Field equipment

- 2ml vial half filled with Ethanol (70-80%)
- Straw, small spatula or tweezers
- Permanent Marker pen
- Kimwipes/tissues
- Plastic bag to collect dirty tissues
- Notebook and pencil

Sample Size

When using universal metazoan markers, the average success for of samples collected randomly can be as low as 15%, even when fresh. Whereas when following these guidelines, sample success was between 50-60%. This success rate is a guide only and may be useful to determine the sample sizes required to achieve the desired number of data points. In large colonies, it is usually possible to get 10 fresh scats per person per hour. Some stages such as incubation, may be lower.

Collecting the sample

Sit on the colony edge and wait for a bird to defecate. This can be time consuming, but will be faster for people that know the behavioural cues. There are distinct behaviours prior to defecation. These include the bird standing, shuffling around usually to face into the wind, before they lean slightly forward to defecate. This is usually followed by a tail shake. Sometimes you will hear the poo, so quickly look for the bird shaking their tail and the direction their bottom is facing to be able to find the scat. For burrowing petrels where observations aren't possible, laying a sheet outside the burrow may enable fresh scats to be collected.

Once a sample is located, collect a small fragment of the non-uric acid portion of the scat (dark part) was using tweezers or a plastic straw. Only a small portion is required (30-50ul or equivalent to half a baked bean). Seabird scats are well mixed, but if the scat is large, take a small amount from multiple parts to ensure the selection is representative of the sample. Avoid the white liquid as this is

primarily urea and doesn't contain dietary DNA. Place the sample in the labelled vial containing 70-80% ethanol. Screw the lid shut and shake the sample to ensure the DNA is well mixed and preserved. Use clean straws/tweezers between samples and bleach at the end of a day.

Storage

Once samples have been collected, keep them out of direct sunlight and try to keep them cool to reduce any degradation. Although they should keep well in the ethanol in the short term, for long periods store them in the freezer at -20°C or if possible -80°C.

Target the dark part of the scat avoiding the runny white urea



<u>Guidelines</u>

- Collect fresh samples where the animal is seen defecating. If this isn't possible, try to collect only samples that still have moisture (recent samples).
- Using a straw, spatula or tweezers collect a small amount of the dark part of the scat (not the white liquid, which is primarily Urea and doesn't contain dietary DNA);
- Place the sample in a 2ml vial containing 70-80% ethanol.
- Tightly close the lid and mix the scat with the ethanol by shaking the tube.
- Clean the spatula or tweezers between scats.
- Take into consideration the seasonal behaviour and feeding ecology of the study animal prior to sample collection.
- Consider the scat substrate type, as contamination from substrate can overwhelm the food DNA signal. Ideally, collect scats from surfaces with minimal sources of DNA contamination (e.g. rocks or ice). If collecting from dirt or vegetation, try to minimise the collection of foreign material and record the substrate (and species where applicable) to cross-check and validate results.
- Collect from animals that are likely to have fed within a short time frame. E.g. not from fasting animals during incubation or when defending nests/territories.
- During incubation, target birds that have returned to the colony within the last 24 hours.
- Samples collected from young chicks may be problematic due to degraded DNA passed on by parents or large amounts of bird DNA. Target scats from adults or older chicks.
- If only a single collection is available and the timing in the season or cohort is not the focus of the dietary question, target the time period with the shortest foraging trip duration and focus on breeding birds.
- Scat collections in the morning may reduce DNA degradation from UV.
- If multiple study sites are used, keep collection protocols and timing consistent between sites

Appendix 6.2: DNA Diet Data Entry Notes for the EG-ABI/EG-BAMM Southern Ocean Trophic Database

This appendix is an amendment to data entry notes originally produced by Ben Raymond for entering conventional dietary diet into the Southern Ocean trophic database. This amendment uses some of the original framework to provide a template for DNA metabarcoding dietary data.

Data sources

Rename your copy of the template file. If you are entering data from a published PDF file, using the same name (but different extension) as the pdf file is helpful.
Enter the details of the data sources into the *source* spreadsheet. Each source (i.e. each paper from which you are entering data) will have its own row in this sheet.

source_id	Your number for this source (start at 1)
details	The bibliographic details for this source (use standard bibliographic format for published papers, e.g. "Hindell M (1988) The diet of the royal penguin Eudyptes schlegeli at Macquarie Island. Emu 88:219–226")
source_notes	Relevant notes about this source – if it's a published paper, paste the abstract here if you can.
filename	The filename of the pdf (if appropriate)
doi	The DOI of the paper or dataset, in the form 10.xxxx/yyyy
citation	The citation to use, if different from the "details" entry

Data records

Enter the DNA data into the *DNA* spreadsheet. Each row in this sheet relates to a single sample observation (i.e. a single instance of something eating something else). There are many columns in this sheet, and you will probably not have information for all of them. Fill out as many as are relevant. A description of each column is given below – for some of them it isn't necessarily obvious what they should contain. In all cases, leaving a cell blank indicates that the information wasn't provided (or can't be entered).
Try and enter data in the finest granularity possible – for example, data might have been collected over several summer seasons. Such data are sometimes provided as individual season summaries as well as a summary over all seasons — use the individual season data in this scenario. If you have access to individual data (i.e. scat samples of individual animals, rather than summaries by groups of animals) then use the individual data.

source_id	Your source number (as entered into the *source* spreadsheet)
location	The name of the location at which the data was collected.
gaz_id	Ignore this – it is an identifier for the placename above, but populated automatically by the loading script
west	The westernmost longitude of the sampling region, in decimal degrees (use negative values for western hemisphere longitudes)
east	The easternmost longitude of the sampling region, in decimal degrees (use negative values for western hemisphere longitudes)

south	The southernmost latitude of the sampling region, in decimal degrees (use negative values for southern hemisphere latitudes)
north	The northernmost latitude of the sampling region, in decimal degrees (use negative values for southern hemisphere latitudes)
altitude_min	The minimum altitude of the sampling region, in metres (if applicable)
altitude_max	The maximum altitude of the sampling region, in metres (if applicable)
depth_min	The shallowest depth of the sampling, in metres (if applicable)
depth_max	The deepest depth of the sampling, in metres (if applicable)
observation_date_start	The start of the sampling period
observation_date_end	The end of the sampling period. If sampling was carried out over multiple seasons (e.g. during January of 2002 and January of 2003), enter the first and last dates as if the sampling was carried out from 1-Jan-2002 to 31-Jan-2003
predator_name	The name of the predator, exactly as it appears in the paper (even if you know it is spelled incorrectly, for example). See the next section for notes on names.
revised_predator_name	The corrected predator name, if applicable. Don't worry if you don't know if a name is correct or not, the loading script will flag names it doesn't recognise.
predator_life_stage	e.g. adult,chick,larva,fingerling
predator_breeding_stage	e.g. brooding, chick rearing, nonbreeding, posthatching
predator_sex	either "male", "female", or "both" (leave empty if unknown)
predator_sample_count	The number of predators for which data are given. If (say) 50 predators were caught but only 20 analyzed, enter 20 here.
predator_total_count	The total number of predators sampled. If (say) 50 predators were caught but only 20 analyzed, enter 50 here.
predator_size_min	The minimum size (not weight) of the predators in the sample
predator_size_max	The maximum size of the predators in the sample
predator_size_mean	The mean size of the predators in the sample
predator_size_sd	The standard deviation of the size of the predators in the sample
predator_size_units	The units of size (e.g. mm)
predator_size_notes	Make a note of what the size value represents. Common entries are "total length", "standard length", or sometimes just "length"
predator_mass_min	The minimum mass (not size) of the predators in the sample
predator_mass_max	The maximum mass of the predators in the sample
predator_mass_mean	The mean mass of the predators in the sample
predator_mass_sd	The standard deviation of the mass of the predators in the sample
predator_mass_units	The units of mass (e.g. "g" or "kg")
predator_mass_notes	Make a note of what the mass value represents (blank implies total body weight)

prey_name	The name of the prey item, exactly as it appears in the paper (even if you know it is spelled incorrectly, for example). See the next section for notes on names.
revised_prey_name	The corrected prey name, if applicable. Don't worry if you don't know if a name is correct or not, the loading script will flag names it doesn't recognise.
prey_is_aggregate	Put a 1 here if this row is an aggregation of other rows in the sheet. For example, there may be a number of individual squid species records, and then an overall squid record that encompasses the individual records. Marking this as an "aggregate" record will avoid double-counting during later analyses. If there is a 0 (or no entry) in this column, it means that this information is not included anywhere else in the data sheet and can be used freely later on when aggregating over taxonomic groups, for example.
sequences_total	This is the total sequence count for the this sample/OTU
DNA_concentration	Sample DNA concentration if recorded in nM/µl
fraction_sequences_by_prey	The fraction of the total food sequences that this prey type made up (e.g. if *Euphausia superba* contributed 50% of the total sequences of prey items, this value would be 0.5) Note: many papers represent very small dietary contributions as "trace" or sometimes "<0.1%". Enter these as -999
fraction_occurrence_all_samples	The number of times this prey item occurred in a predator sample, as a fraction of the number of samples collected (e.g. if *Euphausia superba* occurred in half of the scats collected, this value would be 0.5). Note: many papers represent very small dietary contributions as "trace" or sometimes "<0.1%". Enter these as -999
sample_type	What was DNA extracted from, e.g. scat sample, stomach sample
DNA_extraction_method	DNA stool kit, Maxwell robot, salting out procedure
analysis_type	High-throughput sequencing, cloning, PCR amplification only
sequencing_platform	Ion torrent, Miseq
target_gene	The gene area targeted, either 16S, 12S 18S or CO1 etc
target_food_group	For the 18s region, this may be 'all eukaryotes' for 16s or 12s, this may be 'fish' or 'vertebrates' etc
forward_primer	The sequence of the forward primer used 5'-3'
reverse_primer	The sequence of the reverse primer used 5'-3'
blocking_primer	The sequence of the blocking primer if used 5'-3'
primer_source_id	The paper reference for where the primer was first designed, this should include the PCR conditions, annealing temperature and alignment of the primers
sequence_source_id	The database that contains the sequence data, e.g. Dryad, GenBank etc.
sequence	DNA sequence for OTU or OTU cluster.

Other_methods_applied	Was there any other methods applied to the sample to either improve amplification or block sequences?
qualitative_dietary_importance	If numeric values have not been given (e.g. comments about certain prey in the discussion text), give the qualitative importance here if applicable. Common entries are “minor”, ”major”, ”almost exclusively”, ”incidental”
is_dodgy	Put “Y” here if the data are known to be questionable for some reason. Put the reason in the notes field
is_secondary_data	Enter a *Y* here if the data actually came from another paper and are being reported in this paper as secondary data. Avoid secondary data if possible — go to the original source
is_public	Enter *Y* here unless the data are not to be released for public access yet
entered_by	Your initials
notes	Any other notes

Notes on names

Try and use the correct scientific names in the *revised_*_name* columns if possible. Taxonomy is a horrible thing so if you’re not sure, just enter the name as given in the paper and it will be sorted out during the load process. For unidentified taxa, use the taxonomic name (e.g. Amphipoda, Myctophidae, Decapoda) — use the most specific name without being more specific than the original data.

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